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An engineered *Pseudomonas putida* can simultaneously degrade organophosphates, pyrethroids and carbamates

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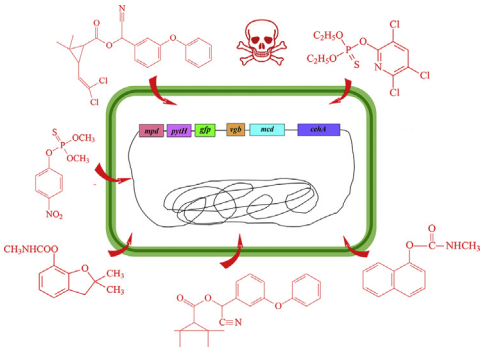
HIGHLIGHTS

- *P. putida* was engineered for simultaneous degradation of three classes of pesticides.
- This GFP-marked strain can be easily tracked by fluorescence during bioremediation.
- VHb enhances the capacity of *P. putida* to sequester oxygen in hypoxic environments.
- This engineered strain is a promising candidate for in situ bioremediation of soil.
- Synthetic biology can serve as a powerful tool to create novel degraders.

GRAPHICAL ABSTRACT



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ABSTRACT

Agricultural soils are often polluted with a variety of pesticides. Unfortunately, natural microorganisms lack the capacity to simultaneously degrade different types of pesticides. Currently, synthetic biology provides powerful approaches to create versatile degraders. In this work, a biosafety strain *Pseudomonas putida* KT2440 was engineered for simultaneous degradation of organophosphates, pyrethroids, and carbamates, enhanced oxygen-sequestering capability, and real-time monitoring by targeted insertion of four pesticide-degrading genes, *vgb*, and *gfp* into the chromosome using a scarless genome-editing method. The resulting recombinant strain, designated as *P. putida* KTUe, could completely degrade 50 mg/L methyl parathion, chlorpyrifos, fenpropathrin, cypermethrin, carbofuran and carbaryl within 30 h when incubated in M9 minimal medium supplemented with 20 g/L glucose. In soil remediation studies, all the tested six pesticides (50 mg/kg soil each) were completely removed in soils inoculated with *P. putida* KTUe within 15 days. Moreover, *Vitreoscilla* hemoglobin (VHb)-expressing *P. putida* KTUe grew faster than *P. putida* KTUd without VHb expression under oxygen-limited conditions, suggesting that VHb may enhance the capability of this recombinant strain to sequester oxygen. Furthermore, the green fluorescence was observed on the *P. putida* KTUe cells, suggesting that this green fluorescent protein (GFP)-marked strain may be tracked by fluorescence during bioremediation. Therefore, this recombinant strain may serve as a promising

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candidate for in situ bioremediation of soil contaminated with multiple pesticides. This work not only underscores the value of *P. putida* KT2440 as an ideal host for bioremediation but also highlights the power of synthetic biology for expanding the degradation capability of natural degraders.

1. Introduction

Pesticides are widely used in agriculture for controlling pests and plant disease vectors, leading to improved crop yields and quality. Extensive use of pesticides poses potential risks to environmental safety and human health. The vast majority of sprayed pesticides reach a destination other than their target species, including non-target species, air, water and soil (Singh and Walker, 2006; Singh, 2009). Many ecosystems are contaminated with pesticides. One survey found that 100% of sampled catchments in Scotland and 75% of sampled aquatic sites in Wales were contaminated with organophosphorus compounds (Boucard et al., 2004). Approximately 3 million poisonings and 300,000 human deaths occur per year owing to OP ingestion (Singh, 2009). Since pesticides tend to be applied in mixtures to crops, multiple pesticide residues are often detected in agricultural soil. Since the coexistence of multiple pesticides can potentiate the toxicity of individual pesticides, the joint toxicity of pesticides poses a serious threat to human lives even if trace pesticides transfer into our body through the food chain (Hernándeza et al., 2013; Zhang et al., 2017).

Microorganisms have evolved their metabolic capacity for efficient degradation of pesticides. To date, various pesticide-degrading microorganisms have been isolated and characterized (Hashimoto et al., 2002; Hayatsu et al., 1999; Ruan et al., 2013; Singh and Walker, 2006; Singh, 2009; Tomasek and Karns, 1989; Trivedi et al., 2016; Wang et al., 2009; Zhai et al., 2012). Several pesticides have been successfully removed from soil using natural degraders such as hexachlorocyclohexane (Lal et al., 2010), chlorpyrifos (Singh et al., 2004) and carbofuran (Jiang et al., 2007). However, there are few reports on simultaneous degradation of different types of pesticides by a natural microorganism. Therefore, the construction of the engineered strains with a broad degradation spectrum is a feasible approach to remediate environment contaminated with multiple pesticides.

In a previous study, a chlorpyrifos-degrading bacterium was isolated from the sludge of the wastewater treating system of a pesticide manufacturer. Furthermore, a *mpd* gene coding for methyl parathion hydrolase (MPH), which exhibits activity against methyl parathion, fenitrothion and chlorpyrifos, was cloned from this strain (Yang et al., 2006). In another study, a novel esterase gene, *pytH*, encoding a pyrethroid-hydrolyzing carboxylesterase (PytH) was cloned from *Sphingobium* sp. strain JZ-1. PytH is a non-specific carboxylesterase that can hydrolyze a variety of pyrethroids (Wang et al., 2009). In addition, a carbofuran hydrolase gene (*mcd*) and carbaryl hydrolase gene (*cehA*) were cloned from *Achromobacter* sp. strain WM111 and *Rhizobium* sp. strain AC100, respectively (Hashimoto et al., 2002; Tomasek and Karns, 1989). Currently, various pesticide-degrading genes could potentially be integrated by transposon or homologous recombination into the chromosome of a host strain for simultaneous degradation of multiple pesticides (de la Peña Mattozzi et al., 2006; Gong et al., 2016a, 2016b; Jiang et al., 2007).

The soil bacterium *Pseudomonas putida* KT2440 is not only certified as GRAS (generally recognized as safe) and endowed with a remarkable metabolic potential, but it also possesses a significant endurance to a large number of organic compounds (Jiménez et al., 2002). Moreover, the whole-genome sequence of *P. putida* KT2440 has been intensively analyzed (Belda et al., 2016; Nelson et al., 2002) and the scarless genome editing methods have been well established in *P. putida* KT2440 (Graf and Altenbuchner, 2011; Luo et al., 2016; Martínez-García and de Lorenzo, 2011), which have paved the way for metabolic pathway engineering in *P. putida* KT2440. Recently, *P. putida* KT2440 has been highlighted as a robust chassis for expression of heterologous metabolic pathways and applied for bioremediation of soil and wastewater (Gong et al., 2016a, 2017).

In this work, the four pesticide-degrading genes (i.e., *mpd*, *pytH*, *mcd* and *cehA*) were integrated into the chromosome of *P. putida* KT2440 by homologous recombination with *upp* as a counter-selectable marker, resulting in an engineered strain capable of simultaneously degrading organophosphates, pyrethroids and carbamates. Moreover, *Vitreoscilla* hemoglobin (VHb) and green fluorescent protein (GFP), which are encoded by *vgb* and *gfp*, respectively, were introduced for enhanced oxygen sequestration and real-time monitoring during bioremediation, respectively. Furthermore, lab-scale soil remediation studies demonstrated the value of this engineered strain for in situ soil bioremediation.

2. Materials and methods

2.1. Chemicals, molecular biology reagents and culture conditions

Methyl parathion, chlorpyrifos, fenpropathrin, cypermethrin, carbofuran, and carbaryl (99% pure analytical grade) were purchased from Alta Scientific Co. Ltd., Tianjin, China. All the other chemical reagents were of analytical grade and purchased from Dingguo Biotechnology Co. Ltd., Tianjin, China. RNA purification kit was purchased from CWBIO Co. Ltd., Beijing, China. RT-PCR kit, TA cloning kit, DNA gel purification kit, and DNA polymerase were purchased from TaKaRa Co. Ltd., Dalian, China.

P. putida strains were routinely cultured at 30 °C in Luria-Bertani medium, pH 7.0 (LB) (Green and Sambrook, 2012) or M9 minimal medium supplemented with 4 g/L glucose, pH 7.0 (M9G) (Gong et al., 2017). If necessary, media were supplemented with 50 μg/mL kanamycin (Kan) or 20 μg/mL 5-fluorouracil (5-FU).

2.2. Construction of the multifunctional pesticide-degrading bacteria by scarless genome editing

The five *P. putida* mutant strains with multiple gene insertions were constructed based on a scarless genome editing strategy using the suicide plasmid pK18mobsacB (Schäfer et al., 1994) in combination with *upp* as a counter-selectable marker. The detailed procedures for the construction of the five *P. putida* mutant strains are described in the supplementary materials. The strains, plasmids, and primers used in this study are listed in Table 1. The detailed information on the inserted exogenous genes is shown in Table 2. The five gene cassettes, including *mpd-pytH*, *mcd*, *cehA*, *gfp*, and *vgb*, were chemically synthesized by Genscript Co. Ltd., Nanjing, China. Both the nucleotide sequences of the five gene cassettes and the modules contained in each cassette are shown in Fig. S1.

Plasmid was transformed into *P. putida* by electroporation using the previously established procedures (Cho et al., 1995). Furthermore, the single-crossover or double-crossover mutant strain was screened on LB agar plates supplemented with 50 μg/mL Kan or 20 μg/mL 5-FU. The detailed screening procedures are described in the supplementary materials. All the constructed mutant strains were validated by PCR detection using the specific primers listed in Table 1. PCR products with the correct size were sent to BGI Inc., Beijing, China for DNA sequencing. Information on PCR detection of the chromosomal insertion of foreign genes is shown in Table S1.

Table 1
Strains, plasmids, and primers used in this study.

Strain, plasmid or primer	Relevant characteristics	Source or reference
Strains		
E. coli		
Trans1 T1	F^-, φ80 (*lacZ*), ΔM15, Δ*lacX74*, *hsdR* (r_K^-, m_K^+), Δ*recA1398*, *endA1*, *tonA*	Transgen
P. putida		
KT2440	Wild type	ATCC 47054
KTU	*upp*-deficient KT2440	This study
KTUa	KT2440 mutant (Δ*upp*, Δ*phaC1*, *mpd*$^+$, *pytH*$^+$)	This study
KTUb	KT2440 mutant (Δ*upp*, Δ*phaC1*, Δ*algA*/*algF*, *mpd*$^+$, *pytH*$^+$, *mcd*$^+$)	This study
KTUc	KT2440 mutant (Δ*upp*, Δ*phaC1*, Δ*algA*/*algF*, Δ*phaZ*/*phaC2*, *mpd*$^+$, *pytH*$^+$, *mcd*$^+$, *cehA*$^+$)	This study
KTUd	KT2440 mutant (Δ*upp*, Δ*phaC1*, Δ*algA*/*algF*, Δ*phaZ*/*phaC2*, Δ*vdh*, *mpd*$^+$, *pytH*$^+$, *mcd*$^+$, *cehA*$^+$, *gfp*$^+$)	This study
KTUe	KT2440 mutant (Δ*upp*, Δ*phaC1*, Δ*algA*/*algF*, Δ*phaZ*/*phaC2*, Δ*vdh*, Δ*fcs*, *mpd*$^+$, *pytH*$^+$, *mcd*$^+$, *cehA*$^+$ *gfp*$^+$, *vgb*$^+$)	This study
Plasmids		
pK18mobsacB	Kan^r, suicide plasmid for gene knockout	Schäfer et al. (1994)
pKU	Kan^r, pK18mobsacB derivative containing *upp* gene	This study
pKUa	Kan^r, pK18mobsacB derivative containing *upp*, *mpd* and *pytH*	This study
pKUb	Kan^r, pK18mobsacB derivative containing *upp* and *mcd*	This study
pKUc	Kan^r, pK18mobsacB derivative containing *upp* and *cehA*	This study
pKUd	Kan^r, pK18mobsacB derivative containing *upp* and *gfp*	This study
pKUe	Kan^r, pK18mobsacB derivative containing *upp* and *vgb*	This study
Primers		
mpd/pytH-1	TGGCCTGGAGCTGAAGAACG	This study
mpd/pytH-2	CAGTGCAACCACCAGGAGTC	This study
mcd-1	AGACTTCCATTGCCAAAGCCCTCAC	This study
mcd-2	ACTGCGCGATGGTCTTCACCGAAAC	This study
cehA-1	CCTGGAAGTCATCGCCTTTGATGTG	This study
cehA-2	GCGGGTTGATGATGCTCTGGATATG	This study
gfp-1	TGGCAGGCGCTGATCTGTTG	This study
gfp-2	TGGCAGATACCCGACTCCAC	This study
vgb-1	GCCCGGACACCACTTTCATC	This study
vgb-2	CTTGGGCATCGCGGTTCAAG	This study
mpd-f	GATGCTGCTGGGCGACTTCGAAATC	This study
mpd-r	AAGGCTTGAACTTGCCGGCCTTCAC	This study
pytH-f	CCGCCCAGTCGCTGACATCCTGGCA	This study
pytH-r	CGGGGAACTCCTTCTGCATCTGACG	This study
mcd-f	GGGCTCAAGATCTATGTGCCCGAAG	This study
mcd-r	CGCCTTGGTCGATTTGGTCCGATAG	This study
cehA-f	CAACATTGCTGCTGGGCGCCAGCCA	This study
cehA-r	TCGAACGGTATCGGCAAGATAGGCC	This study
gfp-f	CACCTACGGCAAGCTGACCCTGAAG	This study
gfp-r	GGACTGGGTGCTCAGGTAGTGGTTG	This study
vgb-f	ATGTTAGACCAGCAAACCATTAACA	This study
vgb-r	TTATTCAACCGCTTGAGCGTACAAA	This study

2.3. RT-PCR assays

P. putida KTUe cells were grown in LB medium in a shaker at 200 rpm and 30 °C for 12 h. The foreign genes are constitutively expressed without the addition of pesticides or other inducers. Extraction of total RNA from *P. putida* KTUe cells was carried out using a RNApure Bacteria Kit (CWBIO, Beijing, China) and the extracted RNA was treated with DNase I at 37 °C for 30 min to eliminate DNA contamination. Quality check of RNA was performed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). cDNA was synthesized with 0.5 μg of total RNA as the template using a PrimeScript RT Master Mix Kit (TaKaRa, Dalian, China). Subsequently, PCR amplification of the specific target genes was carried out on an ABI 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA) with cDNA as the template using a PrimeSTAR HS DNA polymerase (TaKaRa, Dalian, China) and the specific primers. PCR amplification was also performed with genomic DNA, mRNA or ddH_2O as the template under the same conditions. Finally, PCR amplicons were detected by agarose gel electrophoresis. Information on PCR detection of the transcription of foreign genes is shown in Table S1.

2.4. Enzyme activity assays

The cell-free extract of *P. putida* KTUe was used for measuring the activities of four pesticide-hydrolyzing enzymes. Assay for organophosphorus hydrolase activity was performed using methyl parathion or chlorpyrifos as the substrate as described by Yang et al. (2006). Pyrethroid-hydrolyzing carboxylesterase activity was measured with fenpropathrin or cypermethrin as the substrate as described by Wang et al. (2009). Assays for carbofuran hydrolase and carbaryl hydrolase activity were carried out as described by Tomasek and Karns (1989) and Hashimoto et al. (2002). Activities are expressed as units (1 μmol of substrate hydrolyzed per minute) per milligram of protein.

2.5. Pesticide degradation experiments

Cells collected from overnight LB cultures of *P. putida* KTUe were resuspended to $OD_{600} = 1.0$ with M9G (pH 7.0). Subsequently, 5 mL of cell suspensions were inoculated into 95 mL of M9G supplemented with 50 mg/L methyl parathion, chlorpyrifos, fenpropathrin, cypermethrin, carbofuran, and carbaryl and the samples were incubated in a shaker at 200 rpm and 30 °C. The samples (1 mL) were withdrawn every 6 h and extracted thrice with ethyl acetate (1:1, v/v). Finally, the organic layers were pooled and subjected to HPLC and GC analysis. The detection conditions for HPLC and GC are described in the supplementary materials.

2.6. Carbon monoxide-difference spectral analysis

Expression level of VHb was detected using carbon monoxide (CO)-difference spectral analysis as described previously (Zhang et al., 2013). In brief, KTUe cells were harvested by centrifugation, washed with 100 mM potassium phosphate buffer (pH 7.2), resuspended in 20 mL of the same buffer. Cells were disrupted with a sonicator on ice, and the crude extract was centrifuged at 10,000 rpm and 4 °C for 20 min to remove cell debris. VHb in the cell-free extract was reacted with CO

Table 2
Information on six exogenous genes and their chromosomal insertion sites.

Gene	Length (bp)	Amino acid residues	Function	Gene source (GenBank accession no.)	Insertion site
mpd	894	298	Methyl parathion hydrolase	*Stenotrophomonas* sp. strain YC-1 (DQ677027)	PP_5003 (*phaC1*)
pytH	843	281	Pyrethroid-hydrolyzing carboxylesterase	*Sphingobium wenxiniae* strain JZ-1 (FJ688006)	PP_5003 (*phaC1*)
mcd	1983	661	Carbofuran hydrolase	*Achromobacter* sp. strain WM111 (AF160188)	PP_1277/PP_1278 (*algA*/*algF*)
cehA	2385	795	Carbaryl hydrolase	*Rhizobium* sp. strain AC100 (AB069723)	PP_5004/PP_5005 (*phaZ*/*phaC2*)
gfp	720	240	Green fluorescent protein	Plasmid pEGFP-N3 (U57609)	PP_3357 (*vdh*)
vgb	441	147	Hemoglobin	*Vitreoscilla* sp. strain HG1 (AF292694)	PP_3356 (*fcs*)

to produce the CO-VHb complex by exposure to high-purity CO for 2 min. A characteristic peak at 420 nm representing the CO-VHb complex was detected with a UV–VIS spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) using a full-wavelength scanning mode. KTU and KTUd cells were used as the negative controls.

2.7. Imaging bacteria

The KTUe cells were grown to the mid-log phase and harvested. After be washed with PBS buffer (pH 7.2) twice, cells were treated with 10 μM FM4-64/L stain for 15 min. Then, cells were fixed with 2% glycerol on a slice and observed with a Leica TCS SP5 confocal microscope equipped with a Leica 100 × 10 numerical aperture objective lens using the argon laser at 488 nm for GFP excitation.

2.8. Studies for genetic stability and growth kinetics

The strain KTUe was continuously subcultured for 50 generations on LB agar plates at 30 °C. Then, the fiftieth generation subculture was selected for testing the pesticide degradation ability and GFP fluorescence, and the extracted genomic DNA was used as the template for the amplification of four pesticide degradation genes. Procedures for pesticide degradation experiment, fluorescence detection and PCR amplification are the same as those mentioned above. Studies on the growth kinetics of KTUe and KTU cells were performed in M9G medium in a shaking incubator at 200 rpm and 30 °C for 40 h. Cell density was determined by measuring the OD_{600} of the culture broth.

2.9. Soil remediation experiments

The soil samples were collected from the campus of Nankai University, Tianjin, China, which were never exposed to any pesticides before. The soil had a pH of 6.82 and organic matter content of 2.96%. Under aseptic condition, methyl parathion, chlorpyrifos, fenpropathrin, cypermethrin, carbofuran, and carbaryl were added to 100 g of soil samples at the rate of 50 mg/kg for each pesticide. *P. putida* KTUe cells were inoculated into the soil at the rate of 10^6 cells/g and thoroughly mixed with the soil under sterile condition. The soils were incubated at 30 °C for 15 days in the dark. To maintain the soil moisture with 40% of water-holding capacity, distilled water was periodically added to the soil throughout the experiment.

All experiments were performed in triplicate and the soil samples without inoculation were kept as the control. Soil samples (5 g) were withdrawn every 3 days and these pesticides in the soil were extracted with 2 mL of acetone and *n*-hexane (1:1, v/v). The mixtures were centrifuged for 10 min at 3000 rpm and 4 °C and the organic layers were recovered carefully. The extraction process was repeated three times. The organic layers were pooled and dried over anhydrous Na_2SO_4. The organic extracts were passed through a 0.45-μm membrane filter and subjected to HPLC and GC analysis. The detailed procedures for the quantification of these pesticides by GC and HPLC are described in the supplementary materials and were also provided in the previous literatures (Gong et al., 2016a, 2016b).

3. Results

3.1. Construction of a versatile pesticide-degrading strain P. putida KTUe

To create a versatile pesticide-degrading bacterium, in this study, the four pesticide-degrading genes (*mpd*, *pytH*, *mcd* and *cehA*) as well as the *gfp* and *vgb* genes were integrated into the chromosome of a biosafety strain *P. putida* KT2440 using a scarless genome editing method with *upp* as a counter-selectable marker. The stepwise insertion of the above six foreign genes into the *P. putida* chromosome was accomplished by using five homologous recombination processes. The successful construction of the final mutant strain *P. putida* KTUe was confirmed by PCR and DNA sequencing. The DNA fragments with the expected lengths were obtained by PCR with chromosomal DNA of *P. putida* KTUe as the template (Fig. 1), and the nucleotide sequences of the amplified DNA fragments were in agreement with those of the synthetic gene cassettes (Fig. S1).

RT-PCR assays were carried out to verify the transcription of the six foreign genes in *P. putida* KTUe. As a result, the specific DNA bands, which match well with the size of the target fragments, were detected by using agarose gel electrophoresis analysis of PCR products when using cDNA or genomic DNA as the template (Fig. 2). However, the desired products were not obtained by PCR using mRNA or ddH_2O as the template. These results indicated that the foreign genes (i.e., *mpd*, *pytH*, *mcd*, *cehA*, *gfp* and *vgb*) had been transcribed to their respective mRNA molecules.

3.2. Degradation of various pesticides by P. putida KTUe

Both the enzymatic activity assays and pesticide degradation experiments were performed to confirm that the introduced pesticide-degrading genes (i.e., *mpd*, *pytH*, *mcd* and *cehA*) are functionally expressed to produce active enzymes in *P. putida* KTUe. The activities of organophosphorus hydrolase, pyrethroid-hydrolyzing carboxylesterase, carbofuran hydrolase and carbaryl hydrolase were detected in the cell-free extract of *P. putida* KTUe using their corresponding substrates, respectively (Table S2). As expected, the activities were not found in the cell-free extract of *P. putida* KTU. HPLC and GC analysis indicated that each of all the tested six pesticides could be completely degraded in M9G (pH 7.0) by *P. putida* KTUe within 30 h (Fig. 3). In contrast, the decrease in the concentration of the above six pesticides was not detected with those uninoculated controls, indicating that the abiotic degradation processes (e.g., photodegradation and chemical hydrolysis) did not occur in M9G (pH 7.0) under the present experimental conditions. These results suggest that *P. putida* KTUe may be employed for simultaneous degradation of organophosphates, pyrethroids and carbamates.

3.3. Functional expression of VHb and GFP

The presence of VHb within the cell can be confirmed by using the CO-difference spectral analysis, as judged by a characteristic absorption peak at 420 nm because of the formation of the CO-VHb complex (Zhang et al., 2013). In this study, this characteristic 420-nm absorption peak was found when the cell-free extract of *P. putida* KTUe was exposed to CO, while the peak was not observed with the cell-free extract

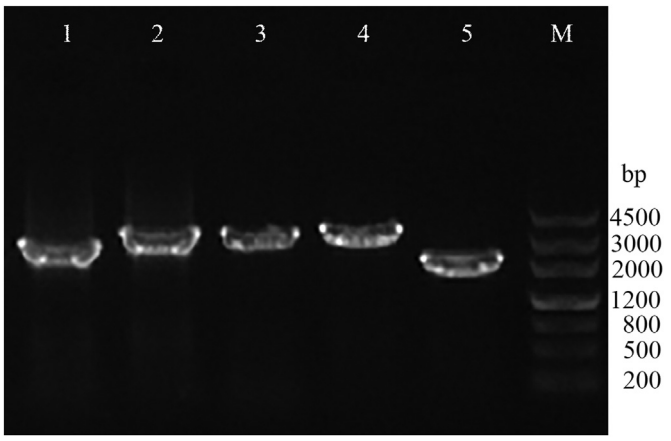

Fig. 1. Detection of multiple gene insertions in *P. putida* KTUe using PCR with chromosomal DNA as the template. Lanes: M, DNA marker; 1, *mpd-pytH*; 2, *mcd*; 3, *cehA*; 4, *gfp*; 5, *vgb*.

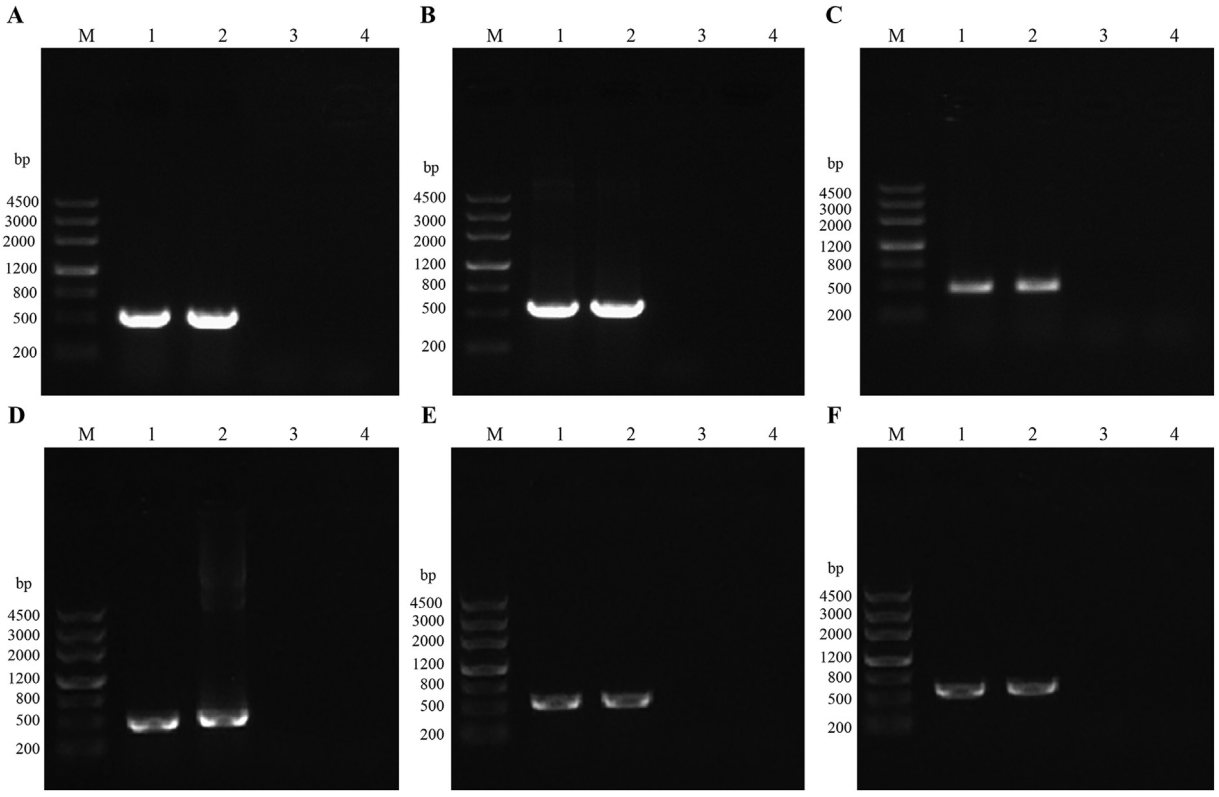

Fig. 2. RT-PCR assays for detecting the transcription of six foreign genes in *P. putida* KTUe. Panels: A, *pytH*; B, *mpd*; C, *vgb*; D, *cehA*; E, *gfp*; F, *mcd*. Lanes: M, DNA marker; 1, PCR reaction using genomic DNA as the template; 2, PCR reaction using cDNA as the template; 3, PCR reaction using mRNA as the template; 4, PCR reaction using ddH_2O as the template.

from the strain KTU or KTUd fed with CO (Fig. 4), which indicated that an active VHb was present in the cell-free extract of *P. putida* KTUe. In this study, VHb-expressing *P. putida* KTUe grew faster than *P. putida* KTUd under oxygen-limited conditions (Fig. S2).

Under a fluorescence microscope, the green fluorescence was observed on the *P. putida* KTUe cells (Fig. 5), indicating that GFP was present in an active form in *P. putida* KTUe.

3.4. Genetic stability and cell growth

The genetic stability of *P. putida* KTUe was confirmed by continuous passage culture. As a result, the fiftieth generation subculture still retained the original degradation capabilities for all the tested six pesticides and emitted bright green fluorescence (data not shown), and the inserted heterologous genes were also obtained by PCR from the subculture (Fig. S3), which indicated that the inserted heterologous genes stably existed in the genome of *P. putida* KT2440.

In this study, we selected the nonessential genes in the genome of *P. putida* KT2440 as the insertion sites of the heterologous genes. To test whether the genome modification inhibits the growth of cells, the growth kinetics of KTUe and KTU cells was compared. As expected, no growth inhibition was observed for the KTUe cells. The KTUe and KTU cells showed the similar growth profiles during a 40-h incubation period and reached a maximum OD_{600} of 2.617 and 2.762 at 28 h, respectively (Fig. S4), which indicated that disruption of the target sites did not affect cellular function and metabolism.

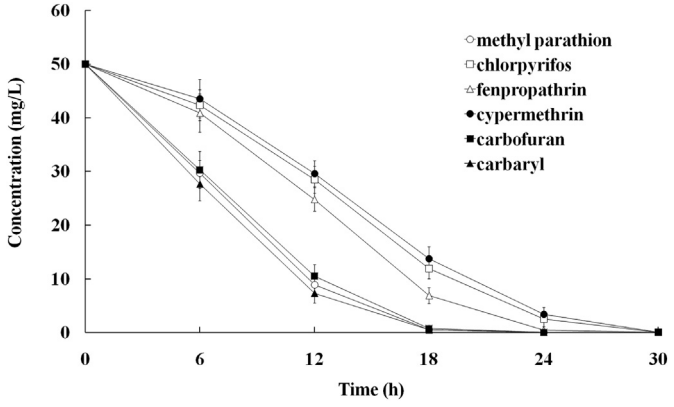

Fig. 3. Simultaneous degradation of organophosphates, pyrethroids and carbamates by *P. putida* KTUe. The degradation experiments were performed at 30 °C with an initial cell density of OD_{600} = 0.05 in M9G supplemented with 50 mg/L methyl parathion, chlorpyrifos, fenpropathrin, cypermethrin, carbofuran, and carbaryl. The data are mean values ± standard deviations from three replicates.

3.5. Bioremediation of soil contaminated with various pesticides by *P. putida* KTUe

In this study, we aim at creating the engineered microorganisms to remediate agricultural soils contaminated with various pesticides;

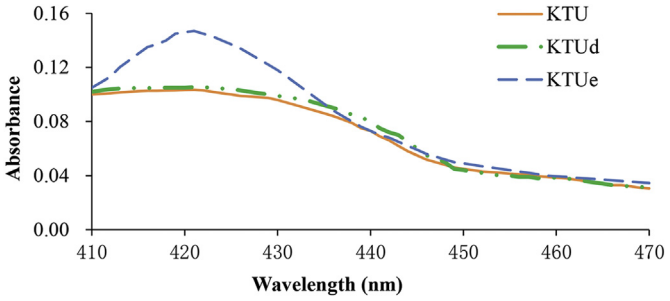

Fig. 4. CO-difference spectral analysis for expression of VHb in *P. putida* KTUe. The cell-free extract showed a characteristic peak at 420 nm due to the binding of CO to VHb. *P. putida* KTU and KTUd were used as the negative controls.

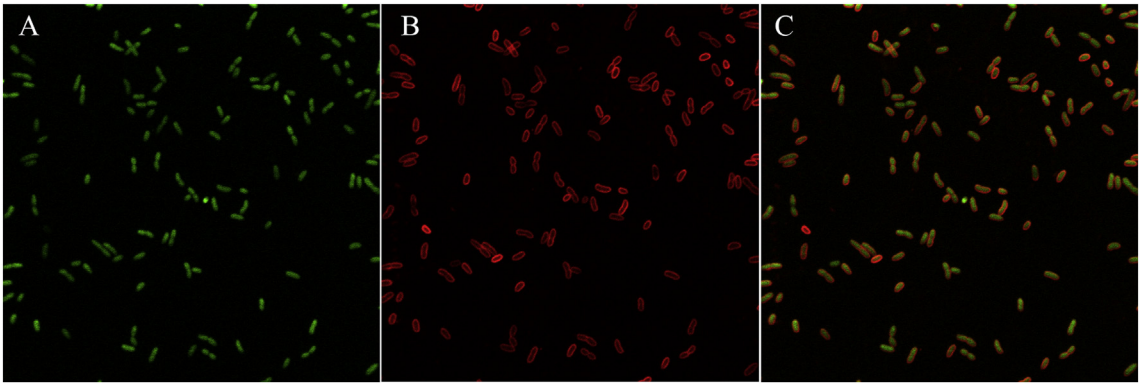

Fig. 5. Detection of GFP fluorescence derived from *P. putida* KTUe using a confocal microscope. Panels: A, Green fluorescence within the cell; B, outline of cell membrane by stain with FM4-64/L; C, panels A and B merged together.

thus, the engineered strain *P. putida* KTUe constructed in this work was investigated for its potential to be used in the cleanup of multiple pesticides in soil. For this purpose, the soil samples collected from the campus of Nankai University were supplemented with six pesticides and inoculated with the strain *P. putida* KTUe at the rate of 10^6 cells/g. During a 15-day period, less than 30% of the amount of each pesticide were degraded in soils without inoculation, possibly resulting from the degradation achieved by the indigenous soil microbial community. In contrast, all the tested six pesticides (50 mg/kg soil each) were completely removed in soils with inoculation within 15 days (Fig. 6). However, the degradation rates for all the tested six pesticides in the soil were much slower than those observed in a liquid culture system (Figs. 3 and 6), which suggested that the recombinant strain *P. putida* KTUe was forced to compete with indigenous microorganisms for limited nutrients in soil.

To test whether the inoculated recombinant strain *P. putida* KTUe is truly responsible for the observed degradation of pesticides during soil bioremediation, we attempted to isolate the strain KTUe from the soil samples at the end of soil remediation experiment. The five bacterial colonies that produced yellow halos on LB agar plates containing 100 mg/L MP were isolated and purified for further identification. All the five isolates, designated as SKe-1, SKe-2, SKe-3, SKe-4 and SKe-5, were identified as the strain *P. putida* KT2440 by the 16S rRNA gene sequencing analysis. Furthermore, all the six heterologous genes (*mpd*, *pytH*, *mcd*, *cehA*, *gfp* and *vgb*) were obtained by PCR from the genomic DNA of the five isolates (data not shown). The successful isolation of the inoculated recombinant strain KTUe suggests that the engineered strain may proliferate well and be more competitive for limited nutrients in the soil microbial community.

4. Discussion

For simultaneous degradation of different types of pesticides by a bacterium, in this work, the four pesticide-degrading genes, *mpd*, *pytH*, *mcd*, and *cehA*, coding for organophosphorus hydrolase, pyrethroid-hydrolyzing carboxylesterase, carbofuran hydrolase, and carbaryl hydrolase, respectively (Hashimoto et al., 2002; Tomasek and Karns, 1989; Wang et al., 2009; Yang et al., 2006), were integrated into the chromosome of *P. putida* KT2440 and functionally expressed to produce four active pesticide-hydrolyzing enzymes. The six pesticides tested in this study could be hydrolyzed by the enzymes. The hydrolytic products of the pesticides cannot be further degraded by *P. putida* KTUe. Pathways for degradation of the six pesticides by *P. putida* KTUe are shown in Fig. S5. Fortunately, the hydrolysis of the pesticides leads to a reduction in mammalian toxicity by several orders of magnitude (Hernándeza et al., 2013), which makes this engineered strain an attractive candidate for large-scale detoxification of various pesticides.

So far, several recombinant strains have been constructed for simultaneous degradation of different types of pesticides. For example, a recombinant strain capable of simultaneously degrading methyl parathion and carbofuran was constructed by random insertion of a *mph* gene into the chromosome of a carbofuran-degrading strain *Sphingomonas* sp. CDS-1, which could use carbofuran as the sole carbon and energy source, using a mini-Tn5 transposon system (Jiang et al., 2007). However, kanamycin resistance gene left on chromosome has potential risks to environmental safety due to its diffusion among bacterial species through horizontal gene transfer. For simultaneous degradation of organophosphates and pyrethroids, a recombinant strain KT-ΔUPP-MP was constructed by targeted insertion of *mpd* and *pytH* into the chromosome using a scarless genome-editing method (Zuo et al., 2015). The recombinant strain *P. putida* KTUe constructed in this study could hydrolyze three types of pesticides via the cleavage of the specific chemical bonds (Fig. S5), but *P. putida* KTUe could not further utilize the hydrolytic products as a carbon source for its growth. Compared with those previously constructed recombinant strains with plasmid-borne pesticide-degrading genes (Wang et al., 2009; Yang et al., 2008; Yang et al., 2017), the degradation rates of the tested pesticides observed with *P. putida* KTUe were slower, but *P. putida* KTUe was endowed with a broader pesticide degradation spectrum. The chromosome-borne degradation genes in *P. putida* KTUe can be maintained in the absence of selection pressure. Moreover, the integration of the degradation genes into the chromosome of *P. putida* neither interrupts cellular functions nor causes host growth defects.

Except for four pesticide-degrading genes, *gfp* and *vgb* genes were also introduced into *P. putida* KT2440. In this study, a strong constitutive promoter J23119 (Shetty et al., 2008) was used to drive the transcription of the six foreign genes in *P. putida* KT2440. RT-PCR assays have demonstrated that the successful transcription of the foreign genes had been achieved by using the promoter J23119 in *P. putida* KT2440.

Previous studies have shown that cell growth, protein synthesis and metabolite yield under oxygen-limited conditions can be enhanced by the heterologous expression of VHb in various bacteria (Stark et al., 2015). VHb can accelerate the transport of oxygen to the respiratory chain, which enhances ATP generation and the efficiency of bacterial aerobic respiration at low oxygen levels (Zhang et al., 2007). In a previous study, improved cell growth coupled with enhanced degradation of 2,4-dinitrotoluene and benzoic acid was observed under oxygen-restricted conditions using *P. aeruginosa* and *Burkholderia* sp. strain DNT with chromosome-borne *vgb* gene (Kim et al., 2005). Sufficient oxygen is regarded as a key factor influencing the efficiency of the aerobic degradation of environmental pollutants. However, oxygen is usually insufficient to support cell growth of the engineered microorganisms during bioremediation of contaminated soil due to the competition from indigenous microorganisms and the fluctuating environmental conditions. The rapid aerobic growth of the engineered microorganisms

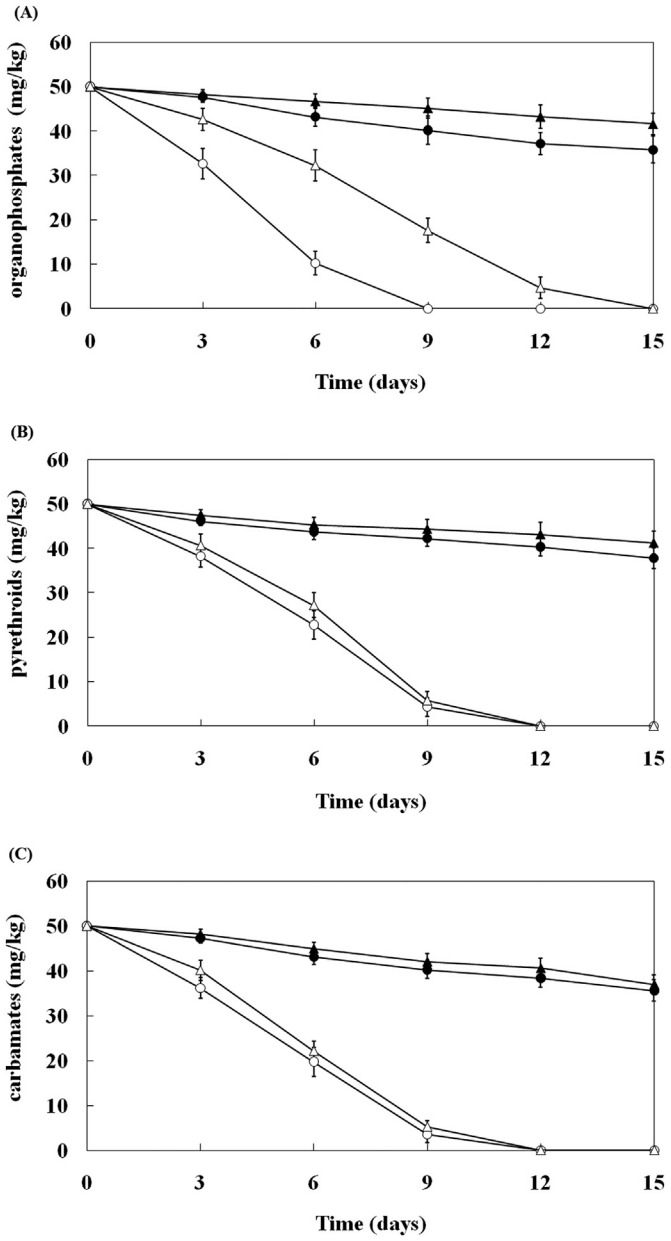

Fig. 6. Simultaneous degradation of organophosphates (panel A), pyrethroids (panel B) and carbamates (panel C) in soil inoculated with *P. putida* KTUe at the rate of 10^6 cells/g. Panel A: ○, methyl parathion, inoculated; Δ, chlorpyrifos, inoculated; ●, methyl parathion, uninoculated; ▲, chlorpyrifos, uninoculated. Panel B: ○, fenpropathrin, inoculated; Δ, cypermethrin, inoculated; ●, fenpropathrin, uninoculated; ▲, cypermethrin, uninoculated. Panel C: ○, carbaryl, inoculated; Δ, carbofuran, inoculated; ●, carbaryl, uninoculated; ▲, carbofuran, uninoculated. The data are mean values ± standard deviations from three replicates.

in hypoxic environments will largely depend on their capability to sequester oxygen. Because VHb may enhance the capacity of *P. putida* KTUe to compete for limited oxygen in oxygen-restricted environments, this bacterium will adapt well to the actual environments.

GFP requires no specific cofactors or exogenous substrates for its fluorescence and can be expressed functionally in various host cells (Chalfie et al., 1994). Moreover, GFP has been used as a molecular marker to monitor the movement and activity of the specific microorganisms in the environment (Errampalli et al., 1999; Larrainzar et al., 2005). Enhanced GFP used in this study exhibits much stronger fluorescence than wild-type GFP and fluoresces after exposure to daylight (Cormack et al., 1996). Therefore, the GFP-marked strain *P. putida* KTUe can be easily tracked by fluorescence during bioremediation.

Currently, combined pollution caused by multiple pesticides can be resolved by using two alternative strategies, including the construction of a multifunctional degrader by introducing various degradation genes into a host strain and the utilization of multiple natural degraders with distinct degradation capabilities (Singh and Walker, 2006; Singh, 2009). However, natural degraders need to be researched intensively for their biosafety prior to practical applications. Members of the genus *Pseudomonas* usually possess a remarkable capacity to degrade a wide variety of toxic substances, and the bacteria are highly active and more competitive in soil (Nikel et al., 2014). For example, a 4-chloro-3-nitrophenol (4C3NP)-mineralizing bacterium, *Pseudomonas* sp. JHN capable of utilizing 4C3NP as a sole carbon and energy source was isolated from a wastewater sample collected from a chemically-contaminated area by an enrichment method (Arora et al., 2014). Also, it was reported that *Pseudomonas* sp. JHN decolourized and biotransformed 4-chloro-2-nitrophenol in the presence of additional carbon source (Arora and Bae, 2014). Previous studies have demonstrated that a biosafety model strain *P. putida* KT2440 possesses diverse catabolic pathways for aromatic compounds (Jiménez et al., 2002). In the present study, *P. putida* KT2440 with available whole-genome sequence and genome-editing methods was used as a chassis for the implantation of various pesticide-degrading genes.

In this work, this homologous recombination process combined with *upp* counter-selection system did not leave any scar in the genome of *P. putida* KT2440, resulting in an unmarked pesticide-degrading strain *P. putida* KTUe. In the future, this current strategy can be utilized for unmarked insertion of various degradation genes into the genome of *P. putida* KT2440 to construct versatile degraders for bioremediation. Synthetic biology can serve as a powerful tool to create novel degraders for bioremediation by assembly of biodegradation pathways using enzymes from different microorganisms (Copley, 2009; Nikel et al., 2014). The application of the versatile degrader *P. putida* KTUe for in situ bioremediation of pesticide-contaminated soil is currently under investigation.

5. Conclusions

Currently, many ecosystems are co-contaminated with different classes of toxic substances. Unfortunately, most of the natural degraders can only degrade individual or one class of contaminants. With the development of synthetic biology, the novel degraders with diverse biodegradation pathways can be created for bioremediation of combined pollution. In this work, the construction of a versatile degrader for simultaneous degradation of organophosphates, pyrethroids and carbamates was achieved by markerless insertion of four pesticide-degrading genes into the genome of a biosafety strain *P. putida* KT2440. This versatile degrader was further engineered for real-time monitoring of its activity and improvement of its oxygen-sequestering capability through the heterologous expression of GFP and VHb. More importantly, the soil remediation studies highlight the value of this versatile engineered strain for in situ bioremediation of pesticide-contaminated soil.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.02.143.

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