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Characterization of a fenpropathrin-degrading strain and construction of a genetically engineered microorganism for simultaneous degradation of methyl parathion and fenpropathrin

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ABSTRACT

A gram-negative fenpropathrin-degrading bacterial strain *Sphingobium* sp. JQL4-5 was isolated from the wastewater treatment sludge of an insecticide factory. Strain JQL4-5 showed the ability to degrade other pyrethroid insecticides, but it was not able to degrade methyl parathion. To enhance its degrading range of substrate, a methyl parathion hydrolase gene (*mpd*) was successfully introduced into the chromosome of strain JQL4-5 with a mini-Tn-transposon system. A genetically engineered microorganism (GEM) named JQL4-5-*mpd* resulted, which was capable of simultaneously degrading methyl parathion and fenpropathrin. Soil treatment results indicated that JQL4-5-*mpd* is a promising multifunctional bacterium in the bioremediation of multiple pesticide-contaminated environments.

1. Introduction

Fenpropathrin (α-cyano-3-phenoxybenzyl-2,2,3,3-tetramethylcyclopropanecarboxylate), a typical pyrethroid insecticide, and methyl parathion (*O*,*O*-dimethyl *O*-4-nitrophenyl phosphorothioate), a common organophosphorus pesticide, are both widely used for the control of agricultural pests in China. However, their residues may cause damages to non-target organisms (Smith and Stratton, 1986; Wu et al., 1999). Fenpropathrin is a potent sodium and potassium channel blocker that produces subtle changes in the function of the channel, causing repetitive neuronal discharge (Soderlund and Knipple, 2002). Methyl parathion inhibits acetyl cholinesterase in an irreversible manner and causes poisoning in humans (Tripathi and Agarwal, 1998; Carlock et al., 1999).

Multiplex tendencies characterize pesticide applications in farming. A number of pesticide mixtures, especially pyrethroid and organophosphorus pesticide mixtures, have been formulated as an improvement over individual pesticides (Moreby et al., 2001). However, the use of pyrethroid and organophosphorus pesticide mixtures can leave multiple pesticide residues in the environment.

Bioremediation is considered to be a major method of degrading contaminants in the environment and can take various forms. Investigation of microbial degradation is useful for the development of insecticide degradation strategies using microorganisms. Bacteria with the ability to degrade methyl parathion have been isolated worldwide (Cui et al., 2001; Liu et al., 2003; Hong et al., 2005). Methyl parathion hydrolase gene, *mpd*, which is responsible for hydrolyzing methyl parathion to *p*-nitrophenol and dimethyl phosphorothioate, has also been cloned from these strains. Sequences are effectively conserved in these strains. Nevertheless, only a few reports on pyrethroid insecticide biodegradation exist so far. Two synthetic pyrethroid-degrading bacteria, *Pseudomonas fluorescens* and *Serratia plymuthica*, have been isolated from sheep dips in India (Grant et al., 2002). *Rhodococcus* sp. CDT3 and *Micrococcus* sp. CPN 1 are two Gram-positive cypermethrin-degrading bacteria, which were isolated in China and India, respectively (Xu et al., 2005; Preeti et al., 2008). During the breakdown process of pyrethroid insecticide, 3-phenoxybenzoate is a transient metabolite in the hydrolysis of the ester linkage. This leads to a loss of insecticidal activity. The compound, 3-phenoxybenzoate, can be further metabolized to yield protocatechuate and phenol. It can even be completely mineralized in *Micrococcus* sp. CPN 1 (Preeti et al., 2008). However, to date, no single microorganism having the ability to

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simultaneously degrade pyrethroid and organophosphorus pesticides has been found. Construction of a genetically engineered microorganism (GEM), which can simultaneously degrade these two kinds of pesticides, would benefit the study and application of bioremediation in multiple pesticide-contaminated environments.

In this study, a fenpropathrin-degrading bacterium, *Sphingobium* sp. JQL4-5, was isolated and characterized. A stable, genetically engineered strain, JQL4-5-*mpd*, capable of simultaneously degrading fenpropathrin and methyl parathion was constructed by random insertion of the methyl parathion hydrolase gene (*mpd*) into the chromosome of strain JQL4-5. Pilot scale bioremediation of soil, co-contaminated by methyl parathion and fenpropathrin, by strain JQL4-5-*mpd* was conducted.

2. Materials and methods

2.1. Chemicals and media

Fenpropathrin (99.2% purity) was purchased from Dalian Ruize Pesticide Co., Ltd. Methyl parathion (98% purity) was purchased from Beijing Kang-Lin Science and Technology Co., Ltd. All the other chemical reagents were of analytical grade and obtained from Nanjing Chemical Reagents No 1. Factory, China.

The mineral salts medium (MSM, pH 7.0) is comprised of the following (g/l): NaCl (1.00), NH_4NO_3 (1.00), K_2HPO_4 (1.50), KH_2PO_4 (0.50), and $MgSO_4 \cdot 7H_2O$ (0.10). The medium was supplemented with fenpropathrin or, when stated, by other compounds as the carbon source. The Luria–Bertani medium (LB, pH 7.0) is composed of the following (g/l): tryptone (10.0), yeast extract (5.0), and NaCl (10.0). For the solid medium, 20.0 g/l of agar was added.

2.2. Isolation and identification of fenpropathrin-degrading strain

To isolate bacteria capable of degrading fenpropathrin, a soil enrichment technique was used. Sludge (1 g) sampled from the wastewater treatment system of an insecticide factory was added to an Erlenmeyer flask (250 ml) containing MSM (100 ml). Fenpropathrin (20 mg/l) was added as a carbon source. The sample was incubated at 30 °C on a rotary shaker at 200 rpm for 7 d. Suspension (1 ml) was transferred to fresh MSM containing fenpropathrin (20 mg/l) and incubated for 7 d. After three rounds of enrichment, the culture was diluted with sterilized 0.1 M phosphate buffer (pH 7.5) by a series of dilutions and plated on MSM plates containing 20 mg/l fenpropathrin. After 3 d of incubation at 30 °C, microbial colonies were visibly observed. The colonies were purified and tested for their fenpropathrin-degrading capability in liquid medium.

The identification of strain JQL4-5 was done according to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and the sequence analysis of its 16S rRNA gene. Genomic DNA was extracted by the high-salt-concentration precipitation method (Miller et al., 1988). The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using standard procedures (Lane, 1991). The PCR product was ligated into the pMD18-T vector (TaKaRa Biotechnology, Dalian, China) and transformed into *Escherichia coli* DH5α. An automatic sequencer (Applied Biosystem, No.3730) was used to determine the 16S rRNA gene sequence. The 16S rRNA gene sequence with 1451 bp was deposited at the GenBank under Accession Number DQ177525. Alignment of the different 16S rRNA gene sequences from the GenBank database was performed using ClustalX 1.8.3 with default settings. Phylogenesis was analyzed by MEGA version 3.0 software. Distances were calculated using the Kimura two-parameter distance model. Unrooted tree was built by the neighbor-joining method. The dataset was bootstrapped 1000 times.

2.3. Analytical method

Methyl parathion in the liquid samples was analyzed as follows. The sample aliquot (1 ml) was extracted with acetone (4 ml). The extracts were dried over anhydrous Na_2SO_4 and evaporated at room temperature. The residual organic material was redissolved in equal volumes of methanol, and 20 μl of the resulting solution was subjected to reversed-phase high-performance liquid chromatography detection (HPLC, 600 Controller, Rheodyne 7725i Manual injector and 247 Dual λ Absorbance Detector; Waters Co., Milford, MA). The separation column (4.6 mm × 250 mm × 5 μm) for the HPLC was filled with Kromasil 100-5C18. The mobile phase contained methanol/water (80:20, v/v) delivered at a flow rate of 1.0 ml/min. For the analysis of methyl parathion in the soil, the soil sample (2 g) was transferred to a 20 ml glass tube, to which anhydrous Na_2SO_4 (2 g) was added. Acetone (10 ml) was added before sealing the tube with a Teflon-lined cap. The sealed tube was vortexed for 30 s and placed in a sonicating bath for 10 min before remixing on the vortex mixer for 15 s. It was then placed in a sonicating bath for another 10 min. The solvent was transferred to a clean, dry glass tube containing activated Florosil™ (1 g, Sigma) and water (0.6 ml). The sealed tube was shaken for 1 min and allowed to stand overnight at ambient temperature. This silica "clean-up" procedure was used to remove interfering humic materials. The extract was finally filtered through a 0.45 μm fiber filter. The extract was treated in the same method as the extract from the liquid sample and subjected to HPLC detection by the above-mentioned method.

Fenpropathrin in liquid samples was analyzed as follows. The sample aliquot (1 ml) was extracted with hexane (3 ml). The extract was dried over anhydrous Na_2SO_4, and then 0.4 μl of the resulting organic phase was analyzed by gas chromatography (GC). GC analysis of fenpropathrin was performed with a SHIMADZU-GC14B equipped with an electron capture 63Ni detector and an SPB-5 column (30.0 m × 0.53 mm × 1.5 μm). The column, injector, and detector temperatures were maintained at 240 °C, 260 °C, and 280 °C, respectively, with a nitrogen flow rate of 1 ml/min. For the analysis of fenpropathrin in soil, the sample was pretreated by the same method as that used for extracting methyl parathion from soil. The resulting extract was subjected to GC detection.

2.4. Construction of GEM

A GEM capable of simultaneously degrading fenpropathrin and methyl parathion was constructed by a triparental conjugation method. *E. coli* SM10 (*thi thr len tonA locy supE recA*::*RP4-2Tc*::*Mu*λ*π R6K*) (λ*pir*) with the R6K-based suicide delivery plasmid, pUT-Km-mpd, was used as the donor strain (*Km*^{r}, 50 mg/l). pUT-Km-mpd provided the IS*50*$_R$ transposase *tnp* gene in *cis*, which is external to the mobile element and whose conjugal transfer to recipients was mediated by RP4 mobilization functions. Mini-Tn5 transposon, located in the pUT-Km-mpd, consisted of a gene-specifying resistance to kanamycin as selection marker and two 19-base-pair I and O Tn5 ends. The functional *mpd* gene fragment with its promoter (1.3 kb) was localized between the two *Not*I sites. *E. coli* HB101 with pRK600 was used as the helper strain (*Cm*^{r}, 20 mg/l). The plasmid pRK600 harbored the gene resistant to chloramphenicol and provided the *tra* function for the mobilization of the pUT-Km-mpd (Jiang et al., 2007). Strain JQL4-5 was used as the recipient strain (*Str*^{r}, 50 mg/l). All the strains were grown overnight in LB medium with the addition of appropriate antibiotics. Cells were collected, washed, and resuspended in LB medium. The donor, helper, and recipient strains were mixed at a ratio of 2:1:3 and loaded on a piece of 0.45 μm cellulose nitrate membrane filter spread on the surface of the LB agar plate. After incubating at 30 °C for 24 h, cells

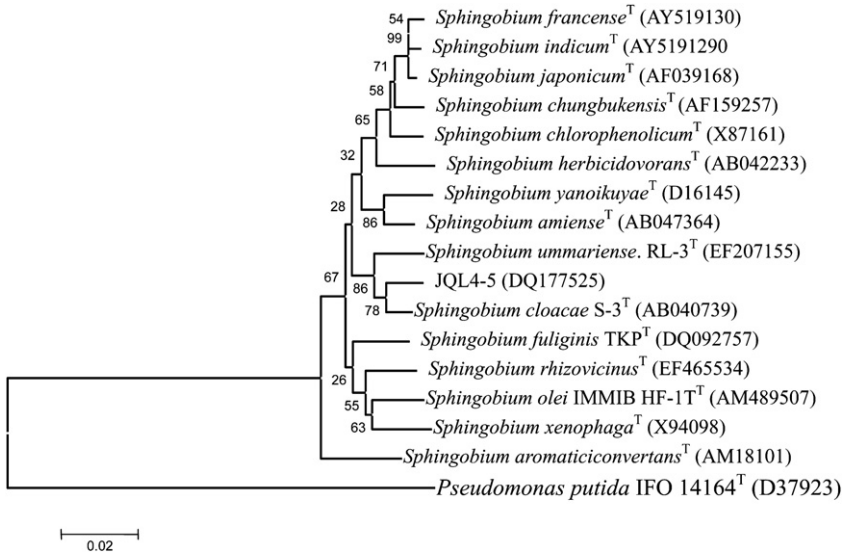

Fig. 1. Phylogenetic tree of strain JQL4-5 based on 16S rRNA gene sequence analysis. Bootstrap values obtained with 1000 repetitions are indicated as percentages at all branches.

were scraped off the filter and resuspended in 1 ml LB medium, and the serial dilutions were spread on an LB plate containing 50 mg/l streptomycin, 50 mg/l kanamycin, and 100 mg/l methyl parathion to select positive exconjugants. Positive exconjugants could hydrolyze methyl parathion to *p*-nitrophenol and produce a clear yellow color around the colony. The insertion of *mpd* gene into the chromosome of the exconjugant was verified by PCR detection with the primer pair, pNot1 (5′-TAGCGGCCGCTCCGTCCAATCTCC-3′) and pNot2 (5′-TAGCGGCCGCTATCACTTGGGGTTG-3′). The genomic DNA of the exconjugant was extracted and used as a template (Jiang et al., 2007).

2.5. Pesticides biodegradation in methyl parathion and fenpropathrin co-contaminated soil

For the soil studies, soil was collected from the campus of the Nanjing Agricultural University, Nanjing, China. This soil was sandy loam with 66% sand, 11% silt, and 16% clay. It contained 2.29% organic matters, 0.127% nitrogen, 0.0948% phosphorus, 2.22 cmol/kg Ca^{2+}, 2.02 cmol/kg Mg^{2+}, 0.13 cmol/kg K^+, and 0.17 cmol/kg Na^+, and had a pH of 6.6. The soil was air-dried, sieved to 2 mm and homogenized before the experiments. JQL4-5-*mpd*, grown in LB medium up to 1×10^8 CFU/ml, was harvested by centrifugation at 6000 × g for 5 min, washed twice by sterilized water, resuspended in MSM medium, and added to contaminated soil at 2×10^6 CFU/g soil. The methyl parathion degrading strain was *Pseudomonas putida* DLL-1 (Liu et al., 2003). and strain JQL4-5 were prepared in the same way as JQL4-5-*mpd*. The *mpd* gene in the chromosome of the GEM strain JQL4-5-*mpd* was originally cloned from the methyl parathion degrading strain, *P. putida* DLL-1. Thus, it was used to compare the methyl parathion degradation ability with strain JQL4-5-*mpd*. Glass beaker (200 ml) microcosms, each containing 100 g soil, were spiked with fenpropathrin (20 mg/kg dry soil) and methyl parathion (50 mg/kg dry soil). To one set of beakers (non-inoculated), MSM medium (4.0 ml, 50% of the water holding capacity of the soil) was added. To another set of beakers (inoculated), MSM medium (4.0 ml) containing the degrading cells was added. The cultures were thoroughly mixed with the soil, and the beakers were covered with perforated aluminum foil and incubated at 30 °C for 15 d under sterile conditions. Distilled water was added every day to compensate for the loss of water by evaporation. Soil samples (5 g) were removed at different time intervals for the analysis of methyl parathion and fenpropathrin concentration.

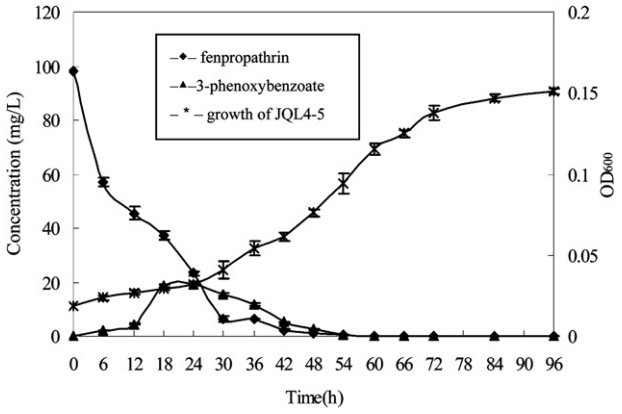

Fig. 2. Degradation of fenpropathrin by strain JQL4-5 (Negative control without inoculation of strain JQL4-5 showed no notable change in pesticides concentration. The data are means± standard deviation for triplicate treatments.) –◆ –fenpropathrin; –▲– 3-phenoxybenzoate;–★– growth of JQL4-5.

3. Results and discussion

3.1. Isolation and identification of the strain JQL4-5

One bacterial strain, JQL4-5, capable of degrading fenpropathrin was isolated. It was a gram-negative, short, rod-shaped bacterium forming buff colonies with obvious sanguine pigment on the LB agar medium after 2–3 d of incubation. The biochemical characteristics were as follows: oxidase, positive; nitrate reductase, positive; Voges–Proskauer test, positive; hydrolysis of starch and gelatin, positive; and methyl red test, negative. Citrate, mannose, acetamide, and L-asparagine maltose could not be utilized as a carbon source by strain JQL4-5. Neither were lactose, benzoic acid (Na salt), and D-mandelic acid. Its 16S rRNA gene sequence was compared with available sequences in the GenBank. JQL4-5 showed

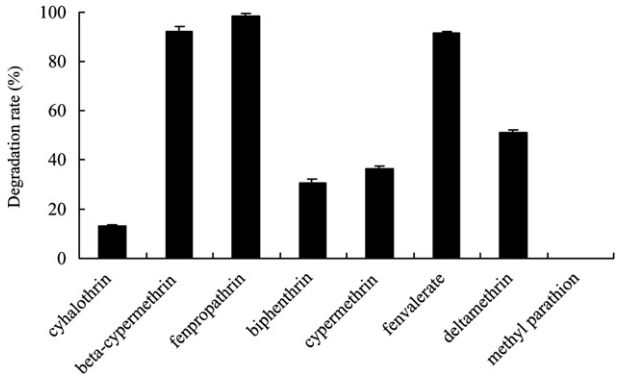

Fig. 3. Degradation of methyl parathion and different pyrethroid pesticides by strain JQL4-5 (Negative control without inoculation of strain JQL4-5 showed no notable change in pesticides concentration. The data are means± standard deviation for triplicate treatments.).

high similarity with the strains of the *Sphingobium* species. It had the highest similarity (98.5%) with the *Sphingobium cloacae* S-3. A phylogenetic tree based on known representatives to describe *Sphingobium* species and other species is presented in (Fig. 1). Based on the above characteristics, strain JQL4-5 was preliminarily identified as *Sphingobium* sp.

3.2. Fenpropathrin degradation by strain JQL4-5

The kinetics of degradation of fenpropathrin and growth of strain JQL4-5 were investigated simultaneously in the MSM media with 100 mg/l fenpropathrin as the carbon source (Fig. 2). The strain underwent a lag phase of growth in the first 24 h. However, the concentration of fenpropathrin reduced quickly in this period, upon the decrease of fenpropathrin. In addition, 3-phenoxybenzoate was accumulated, but its rate of increase was slower than the decrease of fenpropathrin. This showed that 3-phenoxybenzoate could also be degraded by strain JQL4-5. The concentration of 3-phenoxybenzoate increased to a maximum level at 18 h and then decreased until 54 h. Fenpropathrin was completely hydrolyzed at 54 h. The turbidity of the culture (OD_{600}) increased from 0.02 to nearly 0.15 at 96 h. Thus, 3-phenoxybenzoate or other metabolites could be used as carbon sources for the growth of strain JQL4-5. The effects of temperature and initial medium pH on the fenpropathrin degradation by strain JQL4-5 were also investigated. The results showed that the optimal pH and temperature for fenpropathrin degradation were 7–8 and 30 °C, respectively (data not shown).

To examine the degrading effect of strain JQL4-5 on different pyrethroid pesticides and methyl parathion, it was inoculated into the MSM medium separately supplemented with 100 mg/l fenpropathrin, cypermethrin, beta-cypermethrin, biphenthrin, fenvalerate, deltamethrin, cyhalothrin, and methyl parathion. After 48 h of incubation, strain JQL4-5 could degrade more than 90% of fenpropathrin, beta-cypermethrin, and fenvalerate, but showed less degradation activity against deltamelthrin, cypermethrin, biphenthrin, and cyhalothrin. It could degrade 51.2% of deltamelthrin, 36.5% of cypermethrin, 30.6% of biphenthrin, and 13.3% of cyhalothrin, respectively. Strain JQL4-5 could not degrade methyl parathion (Fig. 3). These results might mean that the initial degradation process of pyrethroid pesticide caused by the hydrolysis of the ester band and the specificity of the hydrolyzing activity was broad and pyrethroid molecular structure dependent in strain JQL4-5. The similar activities of pyrethroid hydrolase have been reported in some other strains (Ursel and Juergen, 2001). The pyrethroid molecular structure dependency of pyrethroid hydrolase was recently observed in strain *Sphingobium* sp. JZ-1 (Wang et al., 2009).

3.3. Construction of GEM

The *mpd* gene was randomly inserted into the chromosome of strain JQL4-5 by triparental conjugation. Since the pUT-Km-mpd carrying the $R_{R6K\gamma}$ DNA replicate origin was not able to replicate in strain JQL4-5 (devoid of π replication protein), only transposition events of mini-Tn5 gave resistance to kanamycin-resistant exconjugants. This procedure also allowed a selection of positive exconjugants with *mpd* gene inserted into the chromosome. The *mpd* gene insertion was verified by PCR detection with the primer pair pNot1 and pNot2. Specific 1.3 kb long fragments harboring *mpd* gene could be amplified from all exconjugant genomic DNAs, indicating that the *mpd* gene was located in the chromosome (data not shown). One exconjugant, named JQL4-5-*mpd*, was selected for further study. Its growth and degradation ability was investigated

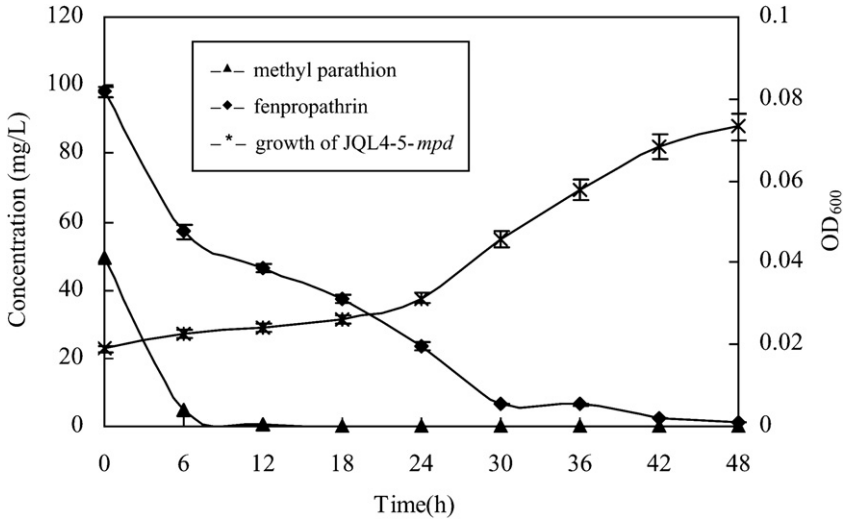

Fig. 4. Degradation of fenpropathrin and methyl parathion by JQL4-5-*mpd* (Negative control without inoculation of strain JQL4-5-*mpd* showed no notable change in pesticides concentration, The data are means± standard deviation for triplicate treatments.) –▲–methyl parathion; –◆– fenpropathrin; –★– growth of JQL4-5-*mpd*.

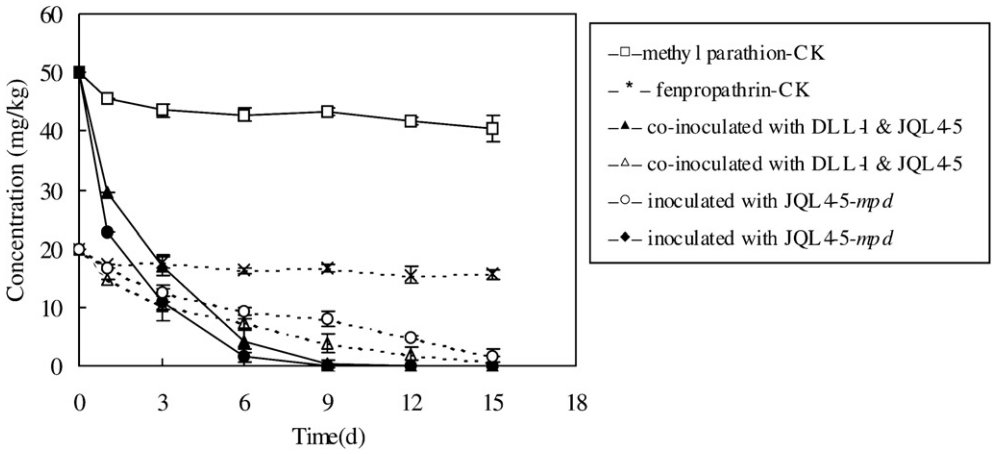

Fig. 5. Biodegradation of methyl parathion and fenpropathrin in co-contaminated soil by different strains (The data are means ± standard deviation for triplicate treatments.) –□– MP-CK; –★– fenpropathrin-CK; –▲–co-inoculated with DLL-1 & JQL4-5; –△– co-inoculated with DLL-1 & JQL4-5; –○– inoculated with JQL4-5-*mpd*; –◆– inoculated with JQL4-5-*mpd*.

in MSM with 100 mg/l fenpropathrin, 50 mg/l methyl parathion, and 50 mg/l kanamycin. Results showed it could simultaneously degrade fenpropathrin and methyl parathion (Fig. 4). Its growth was similar to that of strain of JQL4-5 (Fig. 2).

In order to determine the stability of *mpd* gene in JQL4-5-*mpd*, it was successively cultured at 30 °C for 100 transfers on the LB plate with or without 50 mg/l kanamycin, respectively. Over 200 colonies were randomly selected and determined for their methyl parathion and fenpropathrin-degrading activity. Result showed that all of them had an activity similar to JQL4-5-*mpd*. This indicated that the integration of *mpd* gene cassette into the chromosome of JQL4-5-*mpd* was stable even after the growth for 100 transfers with or without the selection pressure from kanamycin. The *mpd* gene was stable in the chromosome of JQL4-5-*mpd*, similar with the GEM constructed by the same method (Jiang et al., 2007). The key emphasis in this study is on the genetic stability of JQL4-5-*mpd*. Many catabolic genes were located on self-transmissible plasmids, which could be easily lost by horizontal intergeneric exchange in microbial communities in soil (Sayler et al., 1990). This had been well-established for the strains capable of degrading 2,4-dichlorophenoxyacetic acid (2,4-D) herbicide (Top et al., 1998; Newby and Pepper, 2002). Therefore, construction of GEM by introducing catabolic genes with plasmid transferring system into host microorganisms could not settle the genetic stability problem. A GEM strain CDS-pBBR-*mpd* was previously constructed with a plasmid transferring system. It could maintain the genetic stability of the *mpd* gene with a selection pressure from kanamycin but could not maintain it without this selection pressure (Liu et al., 2006). In order to solve this problem, a new recombinant DNA vector mini-Tn5 transposon has been used. This successfully introduced benzyl alcohol dehydrogenase (encoded by *xylB*) and benzaldehyde dehydrogenase (*xylC*) into the chromosome of the host strain 2-chlorobenzoate degraders *Pseudomonas aeruginosa* PA142 and *Pseudomonas aeruginose* JB2 allowing for the construction of a stable GEM for biodegradation of 2-chlorotoluene (Maria and Victor, 2001). This study also proved that mini-Tn5 transposon vector was an effective tool for constructing genetically stable GEM.

3.4. Bioremediation of methyl parathion and fenpropathrin co-contaminated soil

The air-dried natural soil was used in this experiment to simulate the conditions of natural soil because practical bioremediation takes place in natural, non-sterile soil. In this study, only 19.42% of methyl parathion was degraded in 15 d in the control soil, caused mainly by biodegradation of indigenous microorganisms. The co-inoculation of DLL-1 and JQL4-5 could increase the methyl parathion degradation rate sharply. However, inoculation of JQL4-5-*mpd* resulted to a higher methyl parathion degradation rate than a co-inoculation with strains DLL-1 and JQL4-5. Methyl parathion dropped from 50.00 mg/kg to 22.83 mg/kg after 1 d in JQL4-5-*mpd* inoculation treatment, but in the co-inoculation treatment with DLL-1 and JQL4-5, it only dropped to 29.37 mg/kg (Fig. 5). This might be due to the competition between strain DLL-1 and indigenous microorganisms in soil, which influenced the methyl parathion degradation by strain DLL-1. However, methyl parathion in these two treatments was degraded to undetectable levels after 9 d. As for fenpropathrin, indigenous microorganisms degraded less than 25% in the control soil over a 15-d incubation period. The inoculation of JQL4-5-*mpd* resulted in a rapid rate of fenpropathrin degradation, but the degradation rate was a little slower than that of strain JQL4-5. However, there was no distinguishable difference between the final degrading rates between them after 15 d (Fig. 5). This indicated that the GEM strain JQL4-5-*mpd* was able to survive in competition with indigenous microorganisms in natural soil. Thus, it is potentially useful for pesticide bioremediation.

4. Conclusions

In the present study, a GEM, named JQL4-5-*mpd*, possessing simultaneous degradation ability for methyl parathion and fenpropathrin, was constructed by introducing *mpd* into the chromosome of strain JQL4-5 with a mini-Tn-transposon system. It showed similar growth characteristics to the strain JQL4-5. Strain JQL4-5-*mpd* was genetically stable in methyl parathion hydrolase activity even after 100 transfers, with or without the selection pressure. Soil treatment results indicated that JQL4-5-*mpd* is a promising GEM in the bioremediation of multiple pesticide-contaminated environments.

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