Metabolic Engineering of *Pseudomonas putida* for the Utilization of Parathion as a Carbon and Energy Source

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Abstract: *Pseudomonas putida* KT2442 was engineered to use the organophosphate pesticide parathion, a compound similar to other organophosphate pesticides and chemical warfare agents, as a source of carbon and energy. The initial step in the engineered degradation pathway was parathion hydrolysis by organophosphate hydrolase (OPH) to *p*-nitrophenol (PNP) and diethyl thiophosphate, compounds that cannot be metabolized by *P. putida* KT2442. The gene encoding the native OPH (*opd*), with and without the secretory leader sequence, was cloned into broad-host-range plasmids under the control of tac and taclac promoters. Expression of *opd* from the tac promoter resulted in high OPH activity, whereas expression from the taclac promoter resulted in low activity. A plasmid-harboring operons encoding enzymes for *p*-nitrophenol transformation to β-ketoadipate was transformed into *P. putida* allowing the organism to use 0.5 m*M* PNP as a carbon and energy source. Transformation of *P. putida* with the plasmids harboring *opd* and the PNP operons allowed the organism to utilize 0.8 m*M* parathion as a source of carbon and energy. Degradation studies showed that parathion formed a separate dense, non-aqueous phase liquid phase but was still bioavailable. *Biotechnol Bioeng* **78**: 715–721, 2002.
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INTRODUCTION

Organophosphate pesticides and chemical warfare agents are powerful inhibitors of cholinesterase and very toxic to humans (DeFrank, 1991; Serdar et al., 1989). Recent studies have shown widespread pesticide contamination of groundwater and have indicated that consumers are not adequately protected from pesticide contamination (Kolpin et al., 1998; National Research Council Report, 1993). In addition, as signatories to the 1993 Chemical Weapons Convention, the United States has over 30,000 metric tons of chemical warfare agents (many of which are the organophosphorus compounds VX, sarin, and soman) slated to be destroyed via incineration (Mulbry and Rainina, 1998). Not only are there significant political barriers associated with the incineration of the chemical warfare agents, but current operational incineration facilities have also had smokestack emissions of chlorinated organics, heavy metals and chemical warfare agents themselves (Chemical Weapons Working Group).

Given the potential disadvantages of physical and chemical decontamination methods, biodegradation would appear to be a more acceptable alternative. Although hydrolysis using organophosphate triesterases can be effective, toxic products may result. An organism engineered to completely mineralize organophosphates would avoid the generation of potentially toxic hydrolysis products. To demonstrate the potential usefulness of an engineered organism to mineralize organophosphates, the soil microorganism *Pseudomonas putida* was engineered with the pathways for mineralization of the organophosphate pesticide parathion. Parathion was chosen as the model compound for this work because of its availability, low volatility, and relatively low toxicity. *P. putida* was chosen because of its applicability to bioremediation.

The first step in parathion degradation is its hydrolysis to the metabolites diethyl thiophosphate (DETP) and *p*-nitrophenol (PNP) (see Fig.1). Past work on parathion degradation focused on this initial hydrolysis as it results in a reduction of the acute toxicity by approximately 120-fold (Serdar et al., 1989). To our knowledge, no other researchers have attempted to engineer an organism capable of degrading any of the metabolites resulting from organophosphorous hydrolysis. The fate of the resulting metabolites has been left undressed because the metabolites were assumed to be amenable to degradation by other organisms. The metabolite DETP is essentially non-toxic, and previous researchers have isolated cultures capable of using DETP as a source of phosphorous, sulfur, and carbon

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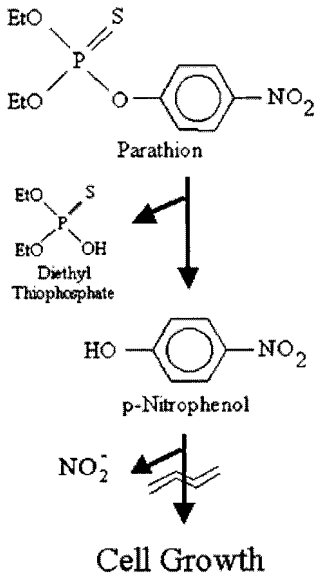

Figure 1. Pathway for parathion metabolism.

(Cook et al., 1978, 1980; Shelton, 1988). The toxicity and fate of PNP is discussed further below.

The bacterial organophosphate hydrolase (OPH, EC 8.1.3.1) catalyzes the hydrolysis of parathion as well as other organophosphorus compounds such as the pesticides coumaphos and diazinon and the chemical warfare agents soman, tabun, and sarin (diSioudi et al., 1999). OPH is encoded by the *opd* gene, which has been cloned and sequenced from both *Pseudomonas diminuta* MG (Serdar et al., 1989) and *Flavobacterium* sp. ATCC 27551 (Mulbry and Karns, 1989); the genes were found to be identical (Harper et al., 1988). OPH is a homodimeric protein with a monomeric molecular weight of approximately 35 kDa (McDaniel et al., 1988; diSioudi et al., 1999; Mulbry et al., 1989). The mature OPH enzyme has two active sites and requires two divalent cation cofactors (Zn^{2+} in the native protein) for each active site (diSioudi et al., 1999). The catalytic mechanism (Lewis et al., 1988) and crystal structure of OPH also have been elucidated (Benning et al., 2001). In addition, several authors have improved the applicability of OPH to chemical warfare agent and pesticide decontamination. The specificity of OPH towards the chemical warfare agents VX and soman was increased using site-directed mutagenesis (diSioudi et al., 1999; Gopal et al., 2000); the rate of hydrolysis of the pesticide paraoxon was increased seven times by successfully expressing OPH on the cell surface as a Lpp-OMP-OPH fusion (Richins et al., 1997).

In order for an organism to use parathion as a source of carbon and energy, the organism must metabolize PNP (see Fig. 1). PNP is a suspected carcinogen and an Environmental Protection Agency priority pollutant (Kadiyala et al., 1998). Therefore, PNP degradation has been studied extensively. Several organisms have been found capable of using PNP as a carbon and energy source, including *Bacillus* (Kadiyala et al., 1998), *Arthrobacter* (Bhushan et al., 2000; Hanne et al., 1993; Jain et al., 1994), *Pseudomonas* (Bang 1997; Prakash et al., 1996; Zylstra et al., 2000), *Burkholderia* (Bhushan et al., 2000), and *Moraxella* (Spain et al., 1979; Spain and Gibson, 1991). Two pathways for PNP degradation have been proposed. One degrades PNP through hydroquinone and the other through 1,2,4-trihydroxybenzene; both pathways converge at β-ketoadipate. Recently, Bang and Zylstra isolated an 18-kb fragment from *Pseudomonas* sp. ENV2030 containing operons encoding PNP transformation through hydroquinone to β-ketoadipate (Bang, 1997; Zylstra et al., 2000). β-Ketoadipate can then be metabolized into trichloroacetic acid

Table I. Strains and plasmids.

Strain or plasmid	Genotype/description	Source or reference
Strains		
E. coli DH5α	F$^-$ Φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44λ$^-$ *thi*-1 *gyr*A96 *rel*A1	Gibco BRL
P. putida KT2440	Plasmid-free mutant of strain mt-2, *rmo*$^-$, *mod*$^+$	(Bagdasarian et al., 1981)
P. putida KT2442	Rifampicin resistant mutant of KT2440	(Franklin et al., 1981)
Plasmids		
pWM513	Native *opd* from *Flavobacterium* sp. ATCC 27551 inserted into the *Pst*I site of pUC19; P_{lac} promoter	(Mulbry and Karns, 1989)
pJK33	Modified *opd* (no leader sequence) inserted into the *Eco*RI and *Bam*HI sites of pUC18; P_{lac} promoter	(Mulbry and Karns, 1989)
pSB337	A 12.7-kb *Xba*I-*Bgl*II fragment (with the native promoters) from *Pseudomonas* sp. ENV2030 encoding PNP transformation to β-ketoadipate inserted into the *Xba*I and *Bam*HI sites of pRK415 (IncP-1, Tet^r)	(Bang, 1997) (Zylstra et al., 2000) (Keen et al., 1988)
pMMB206	A broad-host-range vector with blue/white selection. P_{taclac} *lacl*^{q}, IncP-4, Cm^r	(Morales et al., 1991)
pVLT35	A broad-host-range vector. P_{tac}, *lacl*^{q}, IncP-4, Sm^r	(de Lorenzo et al., 1993)
pAWW01	The modified *opd* from pJK33 inserted into the *Eco*RI and *Bam*HI sited of pMMB206	This work
pAWW02	The native *opd* from pWM513 inserted into the *Pst*I site of pMMB206	This work
pAWW04	The native *opd* from pWM513 inserted into the *Pst*I site of pVLT35	This work

cycle intermediates by the β-ketoadipate pathway in *Pseudomonas*.

In this work, we successfully introduced *opd* and the PNP degradative operons into *P. putida* KT2442, allowing the organism to use parathion as a source of carbon and energy.

MATERIALS AND METHODS

Bacterial Strains and Media

The bacterial strains and plasmids used in this study are listed in Table I. Cultures used for parathion hydrolysis experiments were grown overnight in Luria broth (LB) with the appropriate antibiotic. Expression of isopropyl β-D-thiogalactoside (IPTG)-responsive genes was induced with 100 m*M* (*Escherichia coli*) and 500 m*M* (*P. putida*) IPTG. PNP and parathion-degrading cultures were grown in MOPS (morpholinepropanesulfonic acid)-buffered minimal medium with the appropriate antibiotic (Neidhardt et al., 1974). Antibiotic concentrations were as follows: 100 μg/mL and 1 mg/mL streptomycin (Sm) for *E. coli* and *P. putida*, respectively, 32 μg/mL chloroamphenicol, 50 μg/mL tetracycline (Tet), and 30 μg/mL rifampicin (Rif).

Construction and Transformation of Plasmids

The 1.1-kb OPH-encoding gene, *opd*, was kindly provided by Jeff Karns (USDA) and Wilfred Chen (UC-Riverside). The gene was provided in its native form in the pUC19-based vector pWM513 and in a modified form (the region encoding the N-terminal leader sequence removed) in the pUC18-based vector pJK33 (Mulbry and Karns, 1989). The plasmid containing the 12.7-kb DNA sequence harboring the PNP degradative genes, pSB337, was provided by Gerben Zylstra of Rutgers University.

Plasmids were isolated using the alkaline lysis technique or the Qiagen Mini-Prep kit. The notable exceptions were the broad-host-range RSF1010-based vectors, which were isolated using the boiling lysis technique, as the alkaline lysis procedure resulted in plasmid DNA unamenable to restriction digests.

The modified *opd* was removed from pJK33 by restriction digest with *Bam*HI and *Eco*RI and ligated into the same sites in the broad-host-range plasmid pMMB206 creating pAWW01. Cutting the native form of *opd* from pWM513 with *Pst*I and ligating it into the same site in pMMB206 created the plasmid pAWW02. pAWW04 was created by cutting the native form of *opd* from pWM513 using *Pst*I and ligating it into the same site in the broad-host-range plasmid pVLT35.

Transformation of plasmids into bacterial strains was done using electroporation or triparental mating (Bang, 1997). Electrotransformation into *P. putida* KT2440 and KT2442 was carried out using the method of Cho et al. (1995).

Parathion Hydrolysis Resting Cell Assay

Cultures used for parathion hydrolase assays were grown overnight in LB medium with the appropriate antibiotic and IPTG. Ten milliliters of overnight culture were harvested in exponential phase (OD_{600} less than 1.0), centrifuged at 12,000 × g at 4°C, and resuspended in 5 mL of carbon-free MOPS. The OD_{600} of the resuspended culture was measured, and parathion was added (in the form of 42 m*M* parathion in methanol) to a concentration of 0.8 m*M*. The tubes were shaken at 30°C for 30–120 min, at which time the PNP concentration was quantified. PNP concentration was determined by measuring the absorption at 402 nm on a Beckman DU 640 spectrophotometer (Bang, 1997). The absorption measurements were compared to a standard curve.

PNP Degradation Experiments

P. putida harboring pSB337 was grown overnight at 30°C in LB with Tet. Cells in exponential phase were centrifuged at 12,000 × g at 4°C, the LB media were decanted, and the cells were used to inoculate MOPS medium containing PNP as the carbon and energy source to an initial OD_{600} of 0.05. The culture was grown at 30°C overnight.

To quantify PNP degradation, the overnight PNP-degrading culture was used to inoculate another culture of MOPS medium with PNP to ensure that no residual nutrients from the LB medium were present in the PNP-degrading culture. The OD_{600} of the culture was monitored and the PNP concentration was quantified as above.

Parathion Degradation Experiments

P. putida KT2442 harboring pAWW04 and pSB337 was grown overnight at 30°C in LB with Sm, Tet, and Rif. A small volume of the overnight culture in the exponential phase of growth was harvested as above and used to inoculate MOPS medium with PNP as a carbon and energy source to an OD_{600} of 0.05. The culture was grown on PNP overnight at 30°C and was then used to inoculate MOPS medium containing 0.8 m*M* parathion as the carbon and energy source. Cell growth and PNP concentration were monitored as above.

To quantify parathion concentration, 1 mL of culture was removed at each time point and placed in a 2-mL microcentrifuge tube. Methylene chloride (0.5 mL) was added, and the tube was vortexed for 30 s and then centrifuged at 12,000 × g for 4 min. The organic phase was transferred to a new centrifuge tube and stored at −20°C until it could be analyzed.

Extracted samples were analyzed using a Hewlett-Packard 6890 GC equipped with an electron capture

Table II. Resting-cell parathion hydrolase assays.

Host	Plasmid: Promoter: *opd* gene:	Specific activity (μ*M*/h-OD) pAWW01 P_{taclac} modified	pAWW02 P_{taclac} native	pAWW04 P_{tac} native
E. coli DH5α				
No induction		36.8 ± 2.4	3.8 ± 0.6	10.2 ± 0.5
Full induction		78.8 ± 3.6	10.2 ± 0.6	18.3 ± 1.4
P. putida KT2442				
No induction		1.7 ± 0.0	NA	114 ± 9
Full induction		1.8 ± 0.1	NA	110 ± 13
Activity in native *Flavobacterium* sp. ATCC 27551: 3700 ± 150				

E. coli induced with 100 μ*M* 1PTG; *P. putida* induced with 1 m*M* 1PTG; NA = no activity.

detector (ECD) and an Alltech pesticide capillary column (20 m × 0.53 mm, catalog number 16846). The oven temperature was held constant at 200°C and both the injector and detector temperatures were 240°C. Parathion concentration was determined by comparing peak area of the samples to a standard curve.

RESULTS

OPH Expression

The plasmid pAWW01 was electrotransformed into both *E. coli* DH5α and *P. putida* KT2442. Resting cell hydrolase assays indicated a high level of hydrolase activity and some control over gene expression in *E. coli*, but very little activity in *P. putida* (Table II).

Plasmid pAWW02 was created in an effort to increase hydrolase activity in *P. putida*. pAWW02 is identical to pAWW01 with the exception that the modified form of *opd* was replaced with the native form, which has a secretory leader sequence. pAWW02 was transformed into both *E. coli* DH5α and *P. putida* KT2442, and hydrolase assays revealed the expected order-of-magnitude decrease in activity in *E. coli* (due to the presence of the region encoding the secretory leader sequence), but no activity in *P. putida* (Table II).

The low activity in *P. putida* was hypothesized to result from poor gene expression from P_{taclac}. Therefore, the native form of *opd* was ligated into the broad-host-range vector pVLT35 creating pAWW04. pAWW04 was transformed into both *E. coli* DH5α and *P. putida* KT2442. For *E. coli*, resting cell hydrolase assays indicated a small increase in activity over cells harboring pAWW02. However, *P. putida* cultures harboring pAWW04 showed a dramatic 100-fold increase in activity relative to cells harboring pAWW01 (Table II).

Despite the large increase in activity in *P. putida*, there was no control of *opd* expression from pAWW04 in *P. putida*. In addition, the observed activity in the engineered *Pseudomonas* was still 30-fold lower than the activity measured in the native *Flavobacterium* (Table II).

PNP Degradation

The fragment isolated by Bang and Zylstra encoding the PNP degradative enzymes was provided on the broad-host-range, pRK415-based pSB337. pSB337 contains a 12.7 kb DNA sequence harboring at least three mini-operons encoding PNP transformation to β-ketoadipate; the PNP-degradative genes are expressed in the presence of PNP (Bang, 1997). pSB337 was transformed into both *P. putida* KT2440 and KT2442. Cells harboring pSB337 were capable of utilizing 0.5 m*M* PNP as a carbon and energy source (Fig. 2). The PNP was degraded within 10 h, and the culture had a doubling time of approximately 1.5 h.

A series of PNP degradation studies were conducted in which PNP was provided to triplicate cultures at concentrations ranging from 0.1 m*M* to 1.0 m*M*. Cultures with PNP provided at initial concentrations up to 0.75 m*M* had an initial specific growth rate of approximately 0.45 h^{-1}. However, higher concentrations of PNP severely inhibited growth (specific growth rate approximately 0.2 h^{-1}).

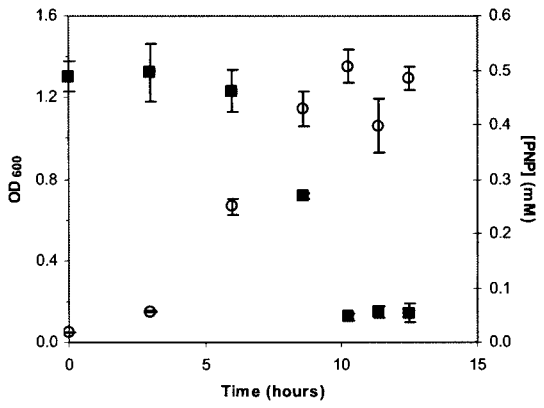

Figure 2. The degradation of PNP by triplicate cultures of *P. putida* KT2440 harboring pSB337. Open circles, OD_{600}; filled squares, PNP concentration. Error bars represent differences in the three cultures.

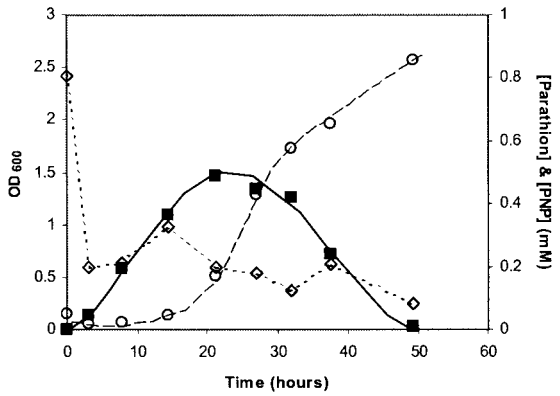

Figure 3. Degradation of parathion by a culture of *P. putida* KT2442 harboring pAWW04 and pSB337. Open circles, OD_{600}; filled squares, PNP concentration; diamonds, parathion concentration.

Parathion Degradation

Parathion utilization as a carbon and energy source was demonstrated by using *P. putida* KT2442 harboring pAWW04 and pSB337. Parathion dissolved in methanol was added to the culture medium to a final concentration of approximately 0.8 m*M*. Degradation studies showed a rapid decrease in parathion concentration, followed first by the appearance and then the disappearance of the metabolite PNP (Fig. 3). An estimated 65% of the provided parathion was metabolized as a source of carbon and energy during the growth experiments.

DISCUSSION

In this work, we successfully introduced *opd* and the PNP degradative operons into *P. putida* KT2442, allowing the organism to use parathion as a source of carbon and energy. The *opd* gene and the PNP degradative operons were introduced into a single organism, as opposed to introducing the *opd* gene into a PNP-degrading bacterium, for several reasons. First, the goal of this work was to demonstrate that multiple metabolic pathways from different organisms could be introduced into a single heterologous host, creating a genetically modified organism capable of degrading a target compound. In addition, assembly of all of the genes encoding the degradative enzymes allows for the entire degradative pathway to be assembled in a variety of different hosts depending on the intended application. Finally, introduction of all of the genes encoding the degradative enzymes into a single organism allows for future optimization of gene expression and the potential to utilize directed evolution to optimize degradation rates and minimize the metabolic burden placed on the cell.

OPH Expression

The OPH encoding gene, *opd*, was transferred in both its native and modified form from the pUC-based plasmids pWM513 and pJK33 to broad-host-range plasmids to allow plasmid replication and gene expression in *P. putida*. OPH produced in *E. coli* DH5α harboring pAWW01 showed very high hydrolase activity. However, activity in *P. putida* KT2442, the organism of interest in this study, was 50-fold lower than in *E. coli*. More importantly, the rate of hydrolysis in *P. putida* KT2442 was too low to allow the organism to utilize parathion as a carbon and energy source after the introduction of the pSB337 plasmid. It was postulated that the low OPH activity in *P. putida* harboring pAWW01 was due to the use of the modified form of *opd*. The modified form of *opd* does not contain the region encoding the N-terminal leader sequence present on the native gene that allows OPH to be membrane associated in the native host (Mulbry and Karns, 1989). Since identical *opd* genes were cloned and sequenced from *Pseudomonas diminuta* MG and *Flavobacterium* sp. ATCC 27551, organisms that are very similar to the *P. putida* being engineered in this work, it was thought that expressing the native *opd* gene in *P. putida* would result in proper processing of the leader sequence. This would allow the OPH to be membrane associated in the engineered Pseudomonad and thus increase hydrolase activity.

The plasmid pAWW02 was constructed to test this hypothesis. Resting cell hydrolase assays on *P. putida* KT2442 harboring pAWW02 showed no OPH activity, indicating that the switch to the native form of the *opd* did not result in the desired increase in activity in *P. putida* as expected. Therefore, the low OPH activity in *P. putida* with pAWW01 or pAWW02 was attributed to poor gene expression from P_{taclac} of pMMB206.

The native *opd* was ligated behind the much stronger P_{tac} of pVLT35 to create pAWW04. Transformation of *P. putida* with pAWW04 resulted in nearly a 100-fold increase in OPH activity relative to cells harboring pAWW01. Thus, the low OPH activity can be attributed to poor gene expression in *P. putida* KT2442 from P_{taclac}. This result is consistent with low gene expression from P_{taclac} observed previously in another Pseudomonad by Morales et al. (1991). However, in this work, a 100-fold increase in gene expression was attributed to the switch from the P_{taclac} to the P_{tac} whereas Morales observed only a five-fold increase. The reason for this difference is unknown but may be associated with the different species of *Pseudomonas* used in this work. Regardless, the P_{taclac} system is not recommended for gene expression in *P. putida*.

Despite the dramatic increase in OPH activity in *P. putida*, it is still 30-fold lower than in the native *Flavobacterium*. Nonetheless, the OPH activity in the engineered organism is still sufficiently high to allow for growth on parathion (see below).

Because pAWW04 was constructed using the native form of *opd*, lysed cells were assayed for OPH activity to determine if the native OPH was being properly processed and membrane associated in the engineered organism. The lysed cell assays showed 47% of the OPH activity being membrane associated in the engineered *P. putida*, very similar to the 38% of total activity that was found to be associated with the membrane fraction in the native *Flavobacterium*. Thus, the native OPH enzyme is most likely being properly processed and is membrane associated in the engineered *P. putida*.

The 38% total activity associated with the membrane fraction in *Flavobacterium* is less than the 64% determined by previous authors (Mulbry and Karns, 1989). This may be attributed to the fact that cultures in this work were isolated in exponential phase after approximately 10 h of growth whereas the previous authors isolated the cultures in post-exponential phase after 48 h of growth.

PNP Degradation

P. putida KT2440 and KT2442 harboring pSB337 were capable of using PNP as a source of carbon and energy (Fig. 2). The rate of PNP utilization by the engineered *Pseudomonas* was similar to the rate observed in the native PNP-degrading *Pseudomonas* sp. ENV2030 (Bang, 1997; Zylstra et al., 2000). This indicates very good expression of the catabolic genes in the PNP degradation operons from pSB337 in *P. putida*.

The growth inhibition of *P. putida* KT2440 harboring pSB337 at a PNP concentration of 1 m*M* is consistent with the inhibition observed in previous studies. PNP-degrading strains of *Sphingomonas* sp. and *Moraxella* sp. were found to be inhibited at PNP concentrations of 0.74 m*M* and 1.1 m*M*, respectively (Leung et al., 1997; Spain and Gibson, 1991). However, the growth inhibition observed in this study occurred at a PNP concentration well below the 6 m*M* concentration at which inhibition was observed in the native *Pseudomonas* sp. ENV2030 from which the operons in pSB337 were taken. This difference is probably attributed to the ability of the native species to withstand high PNP concentrations and is not associated with gene expression in the engineered organism.

Parathion Degradation

P. putida KT2442 harboring pAWW04 and pSB337 was capable of utilizing parathion as a source of carbon and energy. Over the course of the 2-day growth of the culture, parathion was hydrolyzed and PNP first appeared and then disappeared.

It should be noted that there was an initial rapid disappearance of parathion but no corresponding rapid appearance of PNP as would be expected if the observed parathion disappearance was attributed to enzymatic hydrolysis. Instead, the initial disappearance of parathion was the result of parathion being only sparing soluble in water (approximately 20 ppm or 70 μ*M*) and settling out of the aqueous phase in the form of dense, non-aqueous phase liquid spheres. These small spheres were visible at the bottom of the culture tube, and their presence was confirmed by conducting a series of extraction experiments in surfactant-containing media.

Remarkably, despite parathion's tendency to form a separate, non-aqueous phase, it was still bioavailable. Though its solubility in water is just 0.07 m*M*, at least 65% of the added parathion, corresponding to over 0.5 m*M*, was degraded. This result indicates that biodegradation may be an effective treatment strategy in the environment where pesticides are likely to be present in a separate phase.

The slower growth of the engineered Pseudomonad on parathion relative to growth on PNP could be attributed to the need of the culture to first hydrolyze a hydrophobic substrate or the presence of three antibiotics.

CONCLUSIONS

In this work, *P. putida* KT2442 engineered with the genes responsible for parathion hydrolysis and mineralization of PNP was able to use parathion as a source of carbon and energy. The ability of the engineered *Pseudomonas* to degrade parathion is noteworthy, as parathion tends to form a separate organic phase in the culture medium. These results demonstrate the potential of metabolically engineered microorganisms to be used in the remediation and destruction of organophosphorous pesticides and warfare agents.

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