Mineralization of Paraoxon and Its Use as a Sole C and P Source by a Rationally Designed Catabolic Pathway in *Pseudomonas putida*

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Organophosphate compounds, which are widely used as pesticides and chemical warfare agents, are cholinesterase inhibitors. These synthetic compounds are resistant to natural degradation and threaten the environment. We constructed a strain of *Pseudomonas putida* that can efficiently degrade a model organophosphate, paraoxon, and use it as a carbon, energy, and phosphorus source. This strain was engineered with the *pnp* operon from *Pseudomonas* sp. strain ENV2030, which encodes enzymes that transform p-nitrophenol into β-keto adipate, and with a synthetic operon encoding an organophosphate hydrodolase (encoded by *opd*) from *Flavobacterium* sp. strain ATCC 27551, a phosphodiesterase (encoded by *pde*) from *Delftia acidovorans*, and an alkaline phosphatase (encoded by *phoA*) from *Pseudomonas aeruginosa* HN854 under control of a constitutive promoter. The engineered strain can efficiently mineralize up to 1 mM (275 mg/liter) paraoxon within 48 h, using paraoxon as the sole carbon and phosphorus source and an inoculum optical density at 600 nm of 0.03. Because the organism can utilize paraoxon as a sole carbon, energy, and phosphorus source and because one of the intermediates in the pathway (p-nitrophenol) is toxic at high concentrations, there is no need for selection pressure to maintain the heterologous pathway.

Organophosphates have been widely used as nerve agents and pesticides. Examples of these compounds include the pesticides coumaphos and parathion and the nerve gas agents soman, sarin, and VX. These compounds irreversibly inhibit acetylcholine degradation in the human body and can kill quickly by causing persistent and uncontrolled muscle stimulation. Parathion, methyl parathion, and paraoxon, three insecticides, are common model organophosphates because they are much less toxic (in rats, the 50% lethal dose of paraoxon is 1.8 mg/kg, compared to 0.001 mg/kg for VX) and less volatile than many nerve agents. Synthetic in origin, many organophosphates are persistent in the environment and resist degradation by naturally extant microorganisms.

Although incineration and chemical hydrolysis have been widely used to destroy these toxic compounds (20), many microorganisms can detoxify organophosphates by hydrolyzing them using organophosphate acid anhydrases. In general, these enzymes have a broad substrate range and can hydrolyze a number of organophosphate contaminants. While one of these enzymes or some variant can allow the initial detoxification of an organophosphate contaminant, the organism may not degrade the hydrolysis products, some of which are toxic and inhibit bacterial growth (13, 33).

The initial hydrolysis of paraoxon by an organophosphate acid anhydrase (Opd from *Flavobacterium* sp. strain ATCC 27551) to p-nitrophenol (PNP) and diethyl phosphate (DEP) reduces the toxicity 100-fold. Although it is much less toxic than paraoxon, PNP is still classified as a persistent, priority, toxic contaminant (15, 17, 32). In the environment, PNP is degraded by a few microorganisms, most notably *Moraxella* (30) and *Pseudomonas* isolates (27, 34). The genes responsible for degradation of PNP to β-keto adipate, which is further converted to acetyl-coenzyme A (acetyl-CoA) and succinyl-CoA, are located in three small operons in a 13.8-kb region cloned from *Pseudomonas* sp. strain ENV 2030 (10, 29, 35).

The other hydrolysis product, DEP, is relatively inert in the environment and has largely been ignored. *Delftia acidovorans*, one of the few organisms known to use DEP as a sole phosphorus source (5, 6), produces a phosphodiesterase (Pde, encoded by the *pde* gene) that hydrolyzes phosphodiester bonds (31). Expression of the gene, with the aid of a native alkaline phosphatase (PhoA from *Pseudomonas aeruginosa* HN854), enabled *Escherichia coli* and the infectious organism *P. aeruginosa* to use DEP as a phosphate source (8, 31).

Though there is no known single microorganism that is capable of hydrolyzing paraoxon and mineralizing its hydrolysis products as a source of carbon and phosphorus, such a strain not only would reduce the toxicity of the organophosphate but also would prevent the accumulation of potentially toxic hydrolysis products in the environment. Furthermore, the engineered strain would be expected to be genetically stable as it would benefit from the genes introduced to mineralize paraoxon. Today we can use the resources of synthetic biology to create a strain that performs catabolic processes that have not been observed in nature. Here we describe the metabolic engineering of a biosafety *Pseudomonas putida* strain to hydrolyze paraoxon and mineralize the hydrolysis products as a source of carbon and phosphorus (Fig. 1). This strain is a prototype for engineered microorganisms capable of consuming any number of organophosphate contaminants and utilizing...
ing their hydrolysis products for growth, which would be useful for decontamination of equipment contaminated with nerve agents or large-scale destruction of nerve agent stockpiles.

MATERIALS AND METHODS

Strains and media. The strains and plasmids used are listed in Table 1. Luria-Bertani (LB) medium with appropriate antibiotics was used for cloning and in vitro assays. A MOPS (morpholinopropanesulfonate)-buffered minimal medium [40 mM MOPS, 4 mM Tricine, 10 μM FeSO₄, 9.5 mM NH₄Cl, 0.5 μM CaCl₂, 0.521 mM MgCl₂, 50 mM NaCl, 0.276 mM K₂SO₄, 3 × 10⁻⁶ M (NH₄)₆Mo₇O₂₄·4 H₂O, 4 × 10⁻⁷ M H₃BO₃, 3 × 10⁻⁸ M CoCl₂, 1 × 10⁻⁸ M CuSO₄, 8 × 10⁻⁸ M MnCl₂, and 1 × 10⁻⁸ M ZnSO₄, with C and P sources added separately (24)] was used for in vivo assays of paraoxon degradation. This medium allows fine manipulation of phosphate and carbon sources. Paraoxon and diethyl phosphate were obtained from Chem Services, Inc. For solid minimal medium, 2% Noble agar (Difco) was added to the MOPS-buffered minimal medium described above.

Construction of a synthetic operon for paraoxon and DEP hydrolysis. Isolation of the organophosphate hydrolase (Opd) and phosphodiesterase (Pde) genes has been described previously (31, 33). Native Opd was PCR amplified from pWM513 (23) using Pfu Turbo polymerase (Stratagene 600256) and primers opdleadfwd (5'-GGA GGT ACC AGG AGT TAT ATG CAA AC-3') and opdrev (5'-TCC GAA TTCTC ATGA...
CGCCGCGAAGGTCGGTCAACAGACGGC-3') (underlining indicates the ribosome binding site, boldface type indicates the start codon, and italics indicates added KpnI and EcoRI restriction sites in primers opd_fwd and opd_rev, respectively, for cloning into the polylinker in pBBR1MCS2 [14]). The following PCR program was used: 94°C for 1 min, 65°C for 3 min, and 72°C for 2 min for 32 cycles, followed by a 12-min final extension at 72°C. The conserved leader sequence on opd was removed by PCR amplifying only the portion of the gene that did not include the N-terminal leader, using Pfu Turbo polymerase, primers opd mal2 (5′-GGAGGTACCAAGAG GTTTTTATTTAATG CGCAACTCGGA-3′) and opd int (5′-TCCAGGTCCTACGGGCTAGCAATAC-3′) and opd rev (see above), and the same PCR program that was used for the native opd. The truncated opd was present in even-numbered plasmids (Table 1), pde was PCR amplified from pTYB1-pdeCA (31) using Pfu Turbo polymerase and primers pde_fwd (5′-GGAGCTAGT AGGAGGTTTTTATTTAATG CGCAACTCGGA-3′) and pde_rev (5′-TCCAGGTCCTACGGGCTAGCAATAC-3′) (italics indicates added Spel and SacI restriction sites in primers pde_fwd and pde_rev, respectively, for cloning into the polylinker in pBBR1MCS2). phoA from P. aeruginosa HN854 was PCR amplified from genomic DNA using Pfu Turbo polymerase and primers phoA_fwd (5′-ATGGACCATGGATATCCCTGGCTTCTCTTGCGTC TCC-3′) and phoA_rev (5′-GTAGAGCTCTTCA GTCGGAATTACTGTCGCA-3′) (italics indicates an added SacI site). Sequences on all plasmids were confirmed using the UC Berkeley DNA Sequencing Facility.

The PCR-amplified genes were cloned into the polylinker site within the lacZ gene on pBBR1MCS2 [14]. The host plasmid did not carry a functional lacZ, so the genes in the operon were constitutively expressed. The organophosphate hydrolase gene (opd) was cloned into the vector 5′ of the phosphodiesterase gene (pde). The P. aeruginosa HN854 alkaline phosphatase gene (phoA) was toxic when it was expressed in E. coli, so the ligation mixture for any cloning reaction involving expression of this gene was introduced by electroporation into P. putida KT2440, bypassing an E. coli transformation step. In the final plasmid construct, pMM11, the gene order was opd-pde-phoA. Plasmids were transformed into P. putida using an established electroporation method (3). The plasmids were maintained by selection on two antibiotics, 5 μg/mL tetracycline for pSB337 and 50 μg/mL kanamycin for pMM11.

Growth assays and real-time determination of p-nitrophenol concentrations. Cells were typically grown in MOPS medium containing 0.5 or 1 mM paraoxon. Growth assays were typically performed with a 96-well format using a SpectraMax 384 (Molecular Devices, Sunnyvale, CA) or a TECAN SAFIRE (Tecan Ltd., Zurich, Switzerland) to measure optical densities. Cells were grown at 30°C with shaking. Additional assays (Fig. 2) were performed with 50-ml cultures grown in 250-ml baffled 2-L Erlenmeyer flasks shaken at 200 rpm at 30°C. Cell densities are reported below as optical densities at 600 nm (OD₆₀₀).

A method was devised to estimate the real-time concentration of PNP in a 96-well spectrophotometer; this method was modified from a method developed by Miller to measure LacZ activity by the conversion of o-nitrophenyl-β-d-galactopyranoside (22). A similar method involves adaptation of Miller’s assay for a 96-well plate spectrophotometer (9), but this method allows simultaneous determination of both cell density and PNP concentration. Since cells both scatter and absorb light at all wavelengths, the amount of light absorbed by PNP, which is measured primarily at 402 nm and is used to calculate the concentration of PNP, was corrected for the amount of scattering and light absorbed by the cells at 420 nm. Scattering at 402 nm versus scattering at 600 nm was measured for P. putida cells growing in sucinate minimal medium (determined empirically at 1.8) and was used to correct the absorbance at 420 nm for PNP (A₄₂₀) in minimal medium containing cells grown on PNP using the formula

\[ A_{PNP} = A_{420} - (1.8 \times A_{600}) \]

A standard curve for PNP was then constructed for the range of PNP concentrations used in all experiments. The slope (mₛₑₚₚₑ) and intercept (bₛₑₚₚₑ) from this standard curve were used to determine the PNP concentration:

\[ [PNP] = \frac{m_{S_{\text{Succ}}}}{A_{420}} \times (A_{420} - 1.8 \times A_{600}) + b_{S_{\text{Succ}}} \]

There was very little scattering due to cell density in the period while PNP accumulated and was degraded. This method gave an estimate of [PNP] and realistic PNP production rates in an experiment. More precise PNP concentrations were confirmed by repeating the experiments with larger cultures and determining the OD₆₀₀ and [PNP] spectrophotometrically and the [PNP], [DEP], and [paraaxon] by liquid chromatography coupled with triple quadrupole mass spectrometry (LC-MS/MS).

In vitro assays of Opd, Pde, and PhoA activities. The cells in 50 ml of a late-exponential-phase (OD₆₀₀, 0.9) P. putida culture grown in LB medium were pelleted and resuspended in 1.5 ml sonication buffer (50 mM Tris, 10% sucrose, pH 8.0). The cells were disrupted by sonication for 2 min. The cell debris was removed by centrifugation, and the lysate was diluted 100-fold in the sonication buffer. The measured activities were normalized to the total soluble protein in each well, which was determined by the biochromic acid assay (Pierce Bio- sciences, Rockford, Ill.)

(i) Organophosphate hydrolase (Opd) assay. The cell lysate was diluted again fourfold into an Opd assay buffer (100 mM Tris, 1% Triton X-100, 0.8 mM paraoxon; pH 6.92). PNP concentrations were determined colorimetrically at 402 nm against a 0 to 1 mM standard curve at 5-min intervals. The rates of PNP production from paraoxon were calculated and are reported below as the average of four replicates. Specific activity is reported below in μmol PNP produced h⁻¹ mg⁻¹ total protein.

(ii) Alkaline phosphatase (PhoA) assay. The cell lysate was diluted fourfold into a PhoA assay medium (750 mM alkaline buffer solution [Aldrich A9226], 0.8 mM p-nitrophenyl phosphate; pH 10.3). Specific activity was
measured in a fashion similar to the OPD assay, using \( p \)-nitrophenyl phosphate as an ethyl phosphate analog.

(iii) Phosphodiesterase (Pde) assay. The cell lysate was diluted fourfold into a Pde assay medium (750 mM alkaline buffer solution, 0.8 mM bis-\( p \)-nitrophenyl phosphate; pH 10.3). Specific activity was measured in a fashion similar to the OPD assay, using bis-\( p \)-nitrophenyl phosphate as an ethyl phosphate analog. For cells concurrently expressing both \( pde \) and \( phoA \), the PhoA activity was subtracted from the Pde activity, since the product of the Pde reaction is the substrate for the PhoA reaction.

Determination of \( p \)-nitrophenol, paraoxon, and diethyl phosphate concentrations by LC-MS. The growth of \( P. putida \) harboring pSB537 and pMM11 in MOPS minimal medium with 0.5 mM paraoxon as the sole carbon and phosphorus source was monitored over a 6-day period. Two novel LC-MS methods were employed to determine paraoxon, DEP, and PNP concentrations in the high-salt MOPS-buffered medium.

TABLE 2. Multiple reaction monitoring parameters for the detection of paraoxon, PNP, and DEP

<table>
<thead>
<tr>
<th>Compound</th>
<th>Curtain gas pressure (lb/in²)</th>
<th>Collision-activated dissociation gas pressure (lb/in²)</th>
<th>Ion spray voltage (V)</th>
<th>Precursor ion (m/z)</th>
<th>Product ion (m/z)</th>
<th>Declustering potential (V)</th>
<th>Focusing potential (V)</th>
<th>Entrance potential (V)</th>
<th>Collision cell entrance potential (V)</th>
<th>Collision cell energy (V)</th>
<th>Collision cell exit potential (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paraoxon</td>
<td>20</td>
<td>20</td>
<td>5.500</td>
<td>276</td>
<td>220</td>
<td>42</td>
<td>400</td>
<td>2</td>
<td>31</td>
<td>27.8</td>
<td>27.0</td>
</tr>
<tr>
<td>PNP</td>
<td>10</td>
<td>10</td>
<td>-4.500</td>
<td>138</td>
<td>108</td>
<td>-40</td>
<td>-400</td>
<td>-2</td>
<td>-24.2</td>
<td>-25</td>
<td>-25</td>
</tr>
<tr>
<td>DEP</td>
<td>10</td>
<td>10</td>
<td>-4.500</td>
<td>153</td>
<td>79</td>
<td>-40</td>
<td>-400</td>
<td>-2</td>
<td>-22.7</td>
<td>-25</td>
<td>-25</td>
</tr>
</tbody>
</table>

(iv) Liquid chromatography. An Agilent 1100 high-performance liquid chromatography system (Agilent, Palo Alto, CA) was interfaced with an API 2000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). Chromatographic separation, where applicable, was performed using a Magic C\(_{18}\) column (5 \( \mu \)m; 0.3 by 150 mm; 100 Å RP; Michrom Bioresources, Auburn, CA) at 25°C with a flow rate of 10 \( \mu \)l/min.

RESULTS

The primary goal of this study was to engineer an organism that would completely mineralize paraoxon and use it as the sole carbon and phosphorus source for growth. The degradation scheme for accomplishing this goal is shown in Fig. 1. In brief, Opd hydrolyzed paraoxon to DEP and PNP. The PNP was transformed using a known metabolic pathway to \( \beta \)-ketoadipate, which entered the tricarboxylic acid cycle at acetyl-CoA and succinyl-CoA via the action of host-encoded enzymes. The DEF was hydrolyzed using a newly discovered phosphodiesterase (Pde) and alkaline phosphatase (PhoA) to ethanol and orthophosphate, the latter of which could be used as a phosphate source for growth. The enzymes involved in PNP transformation to \( \beta \)-ketoadipate are encoded in a cluster and are from a \( Pseudomonas \) soil isolate. The genes encoding Opd, Pde, and PhoA were placed in a second operon under the control of a constitutive promoter (\( lac \) promoter without a \( lacI \) gene).
TABLE 3. Opd, Pde, and PhoA specific activities in P. putida KT2440 cell lysates

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Gene order in operon</th>
<th>Sp act with 0.8 mM substrate (µg PNP produced h⁻¹ mg⁻¹ protein)</th>
<th>Growth*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Opd</td>
<td>Pde</td>
</tr>
<tr>
<td>pMM11</td>
<td>opd pde phoA</td>
<td>56 ± 3</td>
<td>6.9 ± 1.1</td>
</tr>
<tr>
<td>pMM10</td>
<td>opd pde phoA</td>
<td>82.4 ± 0.9</td>
<td>1.14 ± 0.17</td>
</tr>
<tr>
<td>pMM07</td>
<td>opd pde</td>
<td>35.4 ± 2</td>
<td>1.03 ± 0.18</td>
</tr>
<tr>
<td>pMM06</td>
<td>opd pde</td>
<td>43 ± 7</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>pMM05</td>
<td>phoA</td>
<td>0.46 ± 0.20c</td>
<td>−0.76 ± 0.12c</td>
</tr>
<tr>
<td>pSTopd3</td>
<td>opd</td>
<td>8.89 ± 0.61</td>
<td>−0.065 ± 0.046c</td>
</tr>
<tr>
<td>pSTopd4</td>
<td>opd</td>
<td>24.09 ± 0.43</td>
<td>−0.123 ± 0.177c</td>
</tr>
<tr>
<td>pBBR2pde</td>
<td>pde</td>
<td>0.48 ± 0.05c</td>
<td>2.05 ± 0.34</td>
</tr>
<tr>
<td>pBBR1MCS2</td>
<td>Empty vector</td>
<td>0.14 ± 0.07c</td>
<td>0.4 ± 0.3c</td>
</tr>
</tbody>
</table>

* Growth with 0.5 mM paraoxon as the sole carbon and phosphorus source, coexpressed with pSB337.

a opd contained periplasmic leader sequence.

b Cells do not express the protein.

c pBBR1MCS2 (empty vector containing only phoA).

d pMM11-harboring cells was approximately sixfold greater than that in constructs without the periplasmic signal sequence, pde, and phoA) and was ~30% greater than the specific activity for cells harboring pMM11, which expresses opd’s native periplasmic signal sequence (Table 3). Thus, cells harboring pMM11 accumulated the toxic intermediate PNP more slowly. Cells harboring the empty vector, pBBR1MCS2, had an Opd specific activity of 0.14 µg PNP produced h⁻¹ mg⁻¹ total protein, a level that was barely detectable.

In contrast to the impact of the periplasmic leader sequence on Opd, Pde specific activity was higher in strains in which the Opd had a leader sequence than in strains in which the Opd did not have a leader sequence. The Pde specific activity, measured using bis-p-nitrophenyl phosphate as a DEP analog, for pMM11-harboring cells was approximately sixfold greater than that for pMM10-harboring cells. The only difference between the two constructs was the periplasmic leader sequence on opd in pMM11. The Pde activity from pMM10 and pMM11 was significantly greater than that from all of the other constructs except pBBR2pde (empty vector containing only pde). The phosphodiesterase activity in cells harboring the empty vector alone, pBBR1MCS2, was negligible (Table 3).

Similarly, the PhoA specific activity, measured using p-nitrophenyl phosphate as an ethyl phosphate substrate analog, was greatest in the strain harboring pMM11 (Opd with leader) and was significantly greater than that for any of the other constructs, including pMM10 (without the opd leader sequence) and pMM05 (empty vector containing only phoA). The native alkaline phosphatase activity of P. putida harboring the empty vector was negligible (Table 3).

It is notable that the specific activities of the enzymes involved in DEP hydrolysis were significantly higher than the specific activity of PnpA, the enzyme responsible for the oxidative denitrification of PNP and the first enzyme in the catalytic pathway. The specific activity of PnpA with 0.8 mM substrate was 0.33 ± 0.05 µg PNP produced h⁻¹ mg⁻¹ total protein.

Complete mineralization of paraoxon. The engineered operon containing opd, pde, and phoA (pMM11) was transformed into P. putida with pSB337, which harbors the pnp operon, and paraoxon degradation and growth were monitored in medium in which paraoxon was the sole carbon and phosphorus source. The cultures showed no increase in turbidity for nearly 3 days (Fig. 2a). Nevertheless, the concentration of paraoxon rapidly declined and was undetectable by LC-MS/MS after 24 h (Fig. 2b). The cytotoxic intermediate PNP accumulated in the medium during this time. The average rate of PNP accumulation in the growing cultures was 0.32 mM h⁻¹ OD₆₀₀ unit⁻¹, and the paraoxon consumption rate was approximately equivalent, 0.36 mM h⁻¹ OD₆₀₀ unit⁻¹. This PNP accumulation was most apparent in the first 24 h; the PNP concentrations in the flasks remained high until 54 h. The PNP concentrations, measured both optically (Fig. 2c) and using LC-MS/MS (data not shown), reached a maximal level of 0.38 mM during this period. The cells remained in lag phase while the PNP concentration was maximum and entered logarithmic growth (as determined by LC-MS/MS) did not close completely. Nevertheless, the concentration of paraoxon rapidly declined and was undetectable by LC-MS/MS after 24 h (Fig. 2b). The cytotoxic intermediate PNP accumulated in the medium during this time. The average rate of PNP accumulation in the growing cultures was 0.32 mM h⁻¹ OD₆₀₀ unit⁻¹, and the paraoxon consumption rate was approximately equivalent, 0.36 mM h⁻¹ OD₆₀₀ unit⁻¹. This PNP accumulation was most apparent in the first 24 h; the PNP concentrations in the flasks remained high until 54 h. The PNP concentrations, measured both optically (Fig. 2c) and using LC-MS/MS (data not shown), reached a maximal level of 0.38 mM during this period. The cells remained in lag phase while the PNP concentration was maximum and entered logarithmic growth (as determined by LC-MS/MS) did not close completely. Nevertheless, the concentration of paraoxon rapidly declined and was undetectable by LC-MS/MS after 24 h (Fig. 2b). The cytotoxic intermediate PNP accumulated in the medium during this time. The average rate of PNP accumulation in the growing cultures was 0.32 mM h⁻¹ OD₆₀₀ unit⁻¹, and the paraoxon consumption rate was approximately equivalent, 0.36 mM h⁻¹ OD₆₀₀ unit⁻¹. This PNP accumulation was most apparent in the first 24 h; the PNP concentrations in the flasks remained high until 54 h. The PNP concentrations, measured both optically (Fig. 2c) and using LC-MS/MS (data not shown), reached a maximal level of 0.38 mM during this period. The cells remained in lag phase while the PNP concentration was maximum and entered logarithmic growth. Due to matrix effects on MS quantification, the mass balance for the various components (as determined by LC-MS/MS) did not close completely. These effects are discussed below.

Similar results for mineralization of paraoxon were observed in 100-µl cultures assayed in 96-well plates (data not shown).
*P. putida* mineralized paraoxon up to a concentration of 1.5 mM; higher concentrations resulted in irreversible growth repression by the accumulating PNP. Additionally, when paraoxon was used as a sole carbon and phosphorus source, the cells continued to degrade the pesticide and its hydrolysis products DEP and PNP in the absence of antibiotic selection pressure.

**PNP produced from paraoxon represses cell growth.** It has been reported that PNP is cytotoxic and represses growth (4, 19). When PNP accumulated in the medium to a high concentration, cell growth, and hence paraoxon degradation, ceased. PNP production was measured optically using a 96-well plate format with cultures of cells expressing *opd* (Fig. 3) in MOPS-buffered minimal medium containing glycerol as a carbon source and supplemented with 1 mM paraoxon. The control culture (harboring the empty vector pBBR1MCS2) did not accumulate PNP and grew to an optical density of 0.5 within 24 h. The cells expressing *opd* accumulated PNP to a concentration of 0.6 mM, and their growth was significantly repressed. PNP accumulation was observed for all strains containing the *opd* gene (data not shown). Addition of pSB337 enabled the cells to use this accumulated PNP as a carbon source.

**Growth was phosphate limited.** Because *P. putida* does not naturally produce an alkaline phosphatase, it cannot utilize the ethyl phosphate produced from DEP by the action of Pde. When *phoA* from *P. aeruginosa* was expressed in *P. putida*, it was able to use DEP as a sole phosphate source. However, augmentation of the medium with additional, readily usable phosphate sources (e.g., K2PO4) reduced the lag time to exponential phase from 40 h to 20 h and the maximal accumulation of PNP from 0.3 mM to 0.15 mM (Fig. 4). A similar result was obtained using DEP as a supplemental phosphate source, but the reductions in lag time and PNP accumulation were less pronounced. In all cases, the PNP degradation appeared to be complete by 22 h.

**DISCUSSION**

In this study, we successfully constructed and optimized a novel catabolic pathway for paraoxon and DEP hydrolysis. We introduced this pathway and a second pathway that degrades PNP into the model soil bacterium *P. putida* to enable it to completely mineralize the organophosphate pesticide paraoxon. This rational design of a metabolic pathway for mineralization differs significantly from the traditional bioremediation strategies and represents a new paradigm for laboratory-evolved catabolic pathways (26). One pathway degraded the pesticide into two primary products, DEP and PNP, by the activity of Opd in the synthetic operon. Then, through the activities of Pde and PhoA, the engineered pathway gave *P. putida* the ability to use the DEP as a sole phosphorus source. The other catabolic pathway enabled *P. putida* to use the PNP as a sole carbon and energy source by means of the activities of the five enzymes encoded on pSB337. Paraoxon was completely degraded after 24 h, the PNP was completely consumed after 78 h, and DEP was completely consumed after 142 h. Previous work in our laboratory demonstrated that parathion was used as a carbon source in *P. putida* (33) and in a coculture with *E. coli* (8). In a recent study methyl paraoxon was used as a sole phosphorus source for *E. coli* (21) with a supplemental carbon source. We went a step further and engineered a system to use the organo-

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**FIG. 3.** Production of PNP from paraoxon represses cell growth. *P. putida* harboring either pMM07 (■) or the empty control vector pBBR1MCS2 (○) was grown on a MOPS-buffered minimal medium supplemented with 0.67 mM paraoxon. The error bars indicate standard deviations for four replicate 100-µl cultures. (a) Optical density. (b) PNP concentration.

**FIG. 4.** Growth of and paraoxon degradation by *P. putida* pMM11 pSB337 in the presence of 1 mM DEP or K2PO4. The error bars indicate standard deviations for four replicate 100-µl cultures. (a) OD600. (b) PNP concentration. ○, MOPS-buffered minimal medium with 1 mM paraoxon as the sole C and P source; ■, 1 mM DEP added; ▲, 1 mM K2PO4 added.
phosphate as a phosphorus source, and we condensed the entire bioremediation system into a single microbe.

**Paraoxon hydrolysis and DEP as a sole phosphorus source.** *P. putida* KT2440 harboring pMM11 was capable of using paraoxon as a sole phosphorus source. DEP, a nontoxic and relatively inert intermediate, accumulated in the medium (Fig. 2) until the culture reached logarithmic phase and the cell density increased. The cells then used the DEP as their phosphorus source after the growth repression due to PNP was overcome (see below). The delay was probably due to the catalytic inefficiency of Pde and PhoA compared to the efficiency of Opd (Table 3). The only genetic difference between pMM11 and pMM10, which had the highest Opd hydrolysis rate, was the periplasmic signal sequence on *opd*. We selected the construct pMM11, which had moderate Opd hydrolysis activity and higher Pde and PhoA hydrolysis activities, to slow production of the intermediates DEP and PNP from paraoxon. This allowed a more optimized DEP production rate and growth.

It is notable that there were polar effects in the synthetic *opd*-pde-*phoA* operon. Even when Pde and PhoA were expressed alone, the activities of these enzymes were an order of magnitude lower than that of Opd (Table 3). It is also notable that the periplasmic signal sequence on *opd* had polar effects on the expression of the other two genes in the operon; Opd activity was lower when Opd contained a leader sequence, but Pde and PhoA activities were higher. (*phoA* in this synthetic operon contains its native periplasmic signaling sequence, but *pde* is natively expressed in the cytoplasm.) Perhaps increasing the activities by engineering the active sites on the enzymes or the ribosome binding sites in the mRNA transcript may increase their expression and decrease the DEP accumulation.

When paraoxon is used as a sole carbon and phosphorus source, the molar ratio of carbon to phosphorus is 10:1, whereas the molar ratio for the common soil bacterium is closer to 110:1 (4, 19). Despite the excess of phosphorus in paraoxon, this engineered system appeared to be phosphate limited, at least through the initial stages of the degradation process (Fig. 4). The addition of phosphate as K$_2$PO$_4$ or DEP increased the growth rate and reduced the concentration of PNP that accumulated in the medium. Addition of K$_2$PO$_4$ to the medium increased the growth rate more than addition of DEP increased it; K$_2$PO$_4$ was a more readily available phosphate source as it required no enzymatic cleavage for the cells to utilize it. Increased expression of the phosphoesterases may decrease the phosphate limitation in the early stages of growth.

**PNP from paraoxon as a sole carbon source.** *P. putida* KT2440 expressing pSB337 can use PNP as a sole carbon source, which has been established previously (7, 8, 33, 35). In this case, when *opd* was incorporated into KT2440 harboring pSB337, PNP was generated as paraoxon was hydrolyzed (33). PnpA had a much lower specific activity than Opd, and the resulting accumulation of PNP repressed cellular growth. Degradation of PNP has been identified as a problem previously; various solutions have been devised, such as surface expression of catalytic enzymes (16, 28), increasing inoculum size (34), carbon starvation of the cultures (17), or finding new isolates (15, 18). The concentration of PNP tolerated in this study (up to 0.6 mM) was relatively high compared to concentrations used in these studies, and it was surprising that *P. putida* could tolerate this concentration and still degrade PNP using a natively expressed heterologous system. Regardless, in order for this or any other bioremediation system to be viable, this growth repression has to be overcome. An increased specific activity of PnpA, either due to increased copy number or due to higher catalytic efficiency, will likely decrease the accumulation of PNP.

**New, accurate, and rapid detection procedure for paraoxon and DEP.** Because of the specific constraints in this study, we developed a novel method to analyze DEP and paraoxon that did not involve derivatization. No offline sample preparation method attempted allowed separation of DEP from the other salts in the MOPS-buffered minimal medium; no offline sample preparation beyond dilution was performed. Most methods for analyzing paraoxon (and the analog parathion) from biological samples using LC-MS depend on fractionation of the paraoxon pesticide into primary breakdown products, including DEP and PNP (2, 25). Since we needed to differentiate between paraoxon and its hydrolysis products, a new method was needed. We developed specialized, differential, LC-MS/MS detection procedures to measure paraoxon in positive-ion mode and DEP and PNP simultaneously in negative-ion mode. The best method previously available (11, 12) did not detect DEP under our LC conditions, so we developed a method that relied on the specific detection abilities of triple quadrupole mass spectrometry rather than separation by high-performance liquid chromatography. Despite the fact that the standards were prepared in the same MOPS-buffered minimal medium in which the cells were grown, matrix effects, caused by components of the cell extract, could not be eliminated. Because of these matrix effects, the mass balance of paraoxon, PNP, and DEP did not close completely. Using LC-MS/MS, the concentration of PNP during the accumulation stage was 0.6 mM and the concentration of paraoxon measured initially was 0.2 mM, despite the fact that the paraoxon concentration in the medium was 0.5 mM before inoculation. To account for this, the PNP values reported above are values from optical measurements. Most importantly, however, the same trends were observed using the two methods: the cells degraded paraoxon to undetectable levels within 24 h, PNP within 78 h, and DEP within 142 h.

In conclusion, in this work *P. putida* KT2440 was engineered with two heterologous pathways which allowed it to completely mineralize paraoxon within 142 h and use it as a sole carbon and phosphorus source. The two significant metabolic bottle-necks—growth repression by the intermediate PNP and phosphate limitation due to low bioavailability of DEP—were both resolved by the time that the pesticide was completely mineralized. Additionally, because the organism can utilize paraoxon as a sole carbon, energy, and phosphorus source and because one of the intermediates in the pathway (*p-nitrophenol*) is toxic at high concentrations, there was no need for selection pressure to maintain the heterologous pathway. Further engineering of the system using paraoxon as a sole C and P source is thus self-optimizing. Future studies to address the viability of this system in an environmental setting (e.g., soil or plant detritus) or in an industrial setting may indicate the power of synthetic biology to produce novel strains for bioremediation. With these studies as a basis, engineering organisms for degradation of other PNP-based organophosphates [parathion,
p-nitrophenylethyl(phenyl)phosphinate, and O-ethyl-O-4-nitrophenyl phenylphosphonothioate] should be substantially easier and faster. This study demonstrates the potential of using metabolically engineered organisms in remediation processes for organophosphate pesticides and warfare agents.

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REFERENCES