Improved Degradation of Organophosphorus Nerve Agents and *p*-Nitrophenol by *Pseudomonas putida* JS444 with Surface-Expressed Organophosphorus Hydrolase

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Pseudomonas putida JS444, isolated from *p*-nitrophenol (PNP) contaminated waste sites, was genetically engineered to simultaneously degrade organophosphorus pesticides (OP) and PNP. A surface anchor system derived from the ice-nucleation protein (INP) from *Pseudomonas syringae* was used to target the organophosphorus hydrolase (OPH) onto the surface of *Pseudomonas putida* JS444, reducing the potential substrate uptake limitation. Engineered cells were capable of targeting OPH onto the cell surface as demonstrated by western blotting, cell fractionation, and immunofluorescence microscopy. The engineered *P. putida* JS444 degraded organophosphates as well as PNP rapidly without instability problems associated with the engineered *Moraxella* sp. The initial hydrolysis rate was 7.90, 3.54, and 1.53 μmol/h/mg dry weight for paraoxon, parathion, and methyl parathion, respectively. The excellent stability in combination with the rapid degradation rate for organophosphates and PNP make this engineered strain an ideal biocatalyst for complete mineralization of organophosphates.

Introduction

Organophosphorus compounds (OPs) are widely used as pesticides, insecticides, and chemical warfare agents (*1*–*3*), and their widespread contamination of soil, sediments, and groundwater continues to be a concern today. Due to their extreme toxicity (*4*), there is an urgent need for safe, economical, and reliable methods for detoxification/remediation of these compounds. With the developments in biotechnology, new efforts have been emphasized on the use of microorganisms for the degradation of pollutants rather than disposal. Compared to the potential disadvantage of conventional methods, bioremediation would appear to be more attractive because it is far less disruptive and more cost-effective.

Organophosphorus hydrolase (OPH) isolated from natural soil microorganism *Pseudomonas diminuta* MG and *Flavobacterium* sp. has been shown to hydrolyze a wide range of organophosphorus pesticides (*5*, *6*). Hydrolysis of parathion and methyl parathion, for example, reduced the toxicity by nearly 120-fold and led to the formation of *p*-nitrophenol (PNP) (*7*), which is still classified as a priority pollulant by the U.S. EPA (*8*, *9*). Different bacteria have been isolated to grow on methyl parathion and parathion as the carbon and energy source; however, complete mineralization is usually too slow for practical purposes (*10*, *11*).

To address this problem, OPH has been functionally expressed on the surface of a natural PNP degrader, *Moraxella* sp., using an ice-nucleation protein (INP) anchor, resulting in a single microorganism that is endowed with the capability to rapidly degrade organophosphate pesticides and PNP simultaneously. However, high-level expression of OPH on the surface results in membrane instability and a significant reduction in both PNP degradation and OPH activity.

Several other microorganisms have also been isolated to degrade PNP (*12*). *Pseudomonas putida* JS444 is particularly attractive as it was isolated from PNP-contaminated waste sites and can rapidly degrade PNP through benzoquinone, hydroquinone, maleyl acetate, and β-ketoadipate to tricarboxylic acid intermediates, while releasing nitrite and consuming oxygen (*13*). In this contribution, we demonstrated that OPH could be successfully targeted onto the surface of *Pseudomonas putida* JS444 using the INP anchor, and the resulting recombinant strain is capable of rapidly and simultaneously degrading organophosphate pesticides and PNP without instability problems associated with the engineered *Moraxella* sp.

Materials and Methods

Bacterial Strains and Plasmids. *P. putida* JS444 isolated from activated sludge by selective enrichment with PNP (*13*) was used in this study. The construction of plasmid pPNCO33 containing the INPNC−OPH fusion was described elsewhere (*14*). Strains bearing plasmids were grown in LB medium or minimal salts medium (*15*) supplemented with kanamycin to a final concentration of 50 μg/mL. Cells were grown in 250 mL flasks in a shaker (Innova 4000, New Brunswick Scientific, Edison, NJ) with vigorous agitation (300 rpm) at 30 °C. Expression of the INPNC−OPH fusion was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside).

Transformation of plasmid into *P. putida* JS444 was done using the $MgCl_2$ method (*16*) except for the preparation of competent cells. To prepare competent cells, *P. putida* JS444 was grown overnight in LB medium at 30

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°C and 300 rpm. Overnight cultures were inoculated (OD_{600} = 0.1) in minimal salts medium (*15*) supplemented with 0.2 mM PNP and 0.1% yeast extract until the yellow color of PNP disappeared. At this time additional PNP (0.2 mM) was added and the sequence repeated for three more times. The cells were harvested and incubated in 2 mL of 0.1 M $MgCl_2$ overnight at 4 °C for use.

Cell Fractionation. Following two-day incubation, cells were harvested and resuspended in 25 mM Tris-HCl buffer (pH 8.0). Cells were disrupted by sonication (VirSonic, NY, U.S.A.). The crude extract was then centrifuged for 10 min at 11000*g* to remove any remaining cell debris. The cell-free extract was then centrifuged for 1 h at 115000*g* (Beckman Instruments, CA, U.S.A.) to separate the membrane and soluble fractions (*14*, *16*). The supernatant representing the soluble fraction was retained, and the membrane fraction pellet was resuspended in the same volume of 25 mM Tris-Cl buffer (pH 8.0). A 20 μL sample of total lysate, membrane, and soluble fractions was used for Western blot analysis.

Western Blot Analysis. Samples (10 μL) of OD_{600} = 4.0 cells were mixed with 20 μL of loading buffer (*17*), and the mixtures were boiled for 10 min. Samples of 15 μL were run on a 10% (w/v) acrylamide SDS-PAGE gel. Proteins were then transferred to a nitrocellulose support before incubation with either OPH (*18*) or INP (*19*) antisera. Western blot analysis was preformed using a Bio-Rad Immun-Blot GAR-AP kit (BioRad, Hercules, CA, U.S.A.). Prestained broad-range molecular weight markers were used to estimate protein weights.

Immunofluorescence Microscopy. Following 2 days incubation, cells were harvested and resuspended (OD_{600} = 0.5) in phosphate-buffered saline (PBS) buffer with 3% bovine serum albumin (BSA). Intact cells were then incubated with rabbit anti-OPH antisera (*18*) (1:3000) for 8 h at 4 °C. The cells were washed extensively, resuspended in PBS with secondary antibody (goat anti-rabbit IgG) conjugated with fluorescein isothiocyanate (FITC; Sigma) at a dilution 1:64 and incubated overnight at 4 °C. Prior to microscopy, cells were washed five times with PBS. Photographs were taken using an immunofluorescence microscope (Olympus, Japan).

Organophosphorus Hydrolase Activity Assay. *P. putida* JS444 cells harboring pPNCO33 were grown, harvested, and resuspended in 50 mM citrate−phosphate buffer with 50 μM $CoCl_2$ (pH 8.0). The enzyme activity was measured spectrophotometrically and conducted in a 1.5 mL disposable methacrylate cuvette (Fisher, Tustin, CA, U.S.A.). The cell lysate was prepared by addition of 10 μL of lysozyme (10 mg/mL) and incubated on ice for 1 h, followed by sonication in three pulses of 10 s each. For each assay, 10 μL of 1.0 OD_{600} cells was added to 890 μL of 50 mM, pH 8.0, citrate−phosphate buffer with 50 μM $CoCl_2$ and 100 μL of 20 mM paraoxon (Sigma). Changes in absorbance (412 nm) were measured for 3 min at 37 °C. Activities were expressed in U (micromoles of paraoxon hydrolyzed per hour per mg dry weight) (e_{412} = 16 500 M^{-1} cm^{-1} for *p*-nitrophenol at pH 8.0). Similar whole cell activity measurements were conducted with *P. putida* JS444 harboring pPNCO33 (*14*, *16*).

Simultaneous Degradation of Organophosphates and PNP. *P. putida* JS444 carrying pPNCO33 was inoculated into LB medium with 50 μg/mL kanamycin and incubated overnight on a gyratory incubator shaker at 30 °C and 300 rpm. Subsequently, cells were inoculated (OD_{600} = 0.1) in minimal salts medium (*15*) supplemented with 1 mM IPTG, 0.2 mM PNP, 0.1% yeast extract, and 50 μg/mL kanamycin and incubated at 30 °C and 300 rpm until the yellow color of PNP disappeared. At this time additional PNP (0.2 mM) was added and the sequence repeated for three more times. The cells were harvested using a refrigerated centrifuge (Beckman Instruments, CA) at 4 °C, followed by washing with buffer (50 mM, pH 8.0, citrate−phosphate buffer with 50 μM $CoCl_2$) twice. The pellet was resuspended in the same buffer and stored in refrigerator overnight until use. For the organophosphates and PNP degradation test, 0.4 mM paraoxon, parathion, or methyl parathion was added to cell suspension (OD_{600} = 0.4). Samples were taken at different time points, diluted with 900 μL of 50 mM citrate−phosphate buffer (pH 8.0), and measured for PNP formation and the residual organophosphate concentration using methods as described before (*20*, *21*).

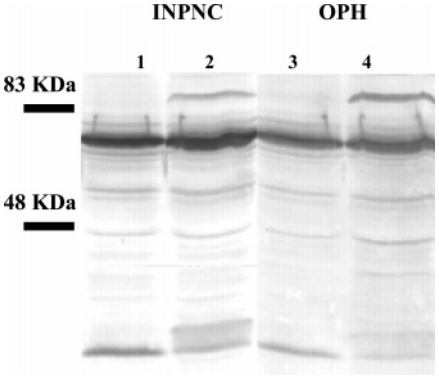

Figure 1. Expression and localization of INPNC−OPH fusions. Western blot analysis with OPH antisera or INPNC antisera. Whole cell lysates of *Pseudomonas putida* JS444 harboring pPNCO33 (2, 4) or pVLT33 (1, 3) were used.

Results and Discussion

Surface Expression and Localization of OPH. An ice-nucleation protein (INP) anchor which has been used to target proteins to the cell surface of *Escherichia coli* (*19*, *20*, *22*−*24*), *Salmonella* (*25*), *Moraxella* sp. (*14*), and *P. putida* KT2440 (*16*) was used in this study. To target OPH onto the surface of *P. putida* JS444, the shuttle vector pPNCO33 carrying the *inpnc-oph* fusion was introduced (*14*). Expression of full-size INPNC−OPH fusions was probed with both OPH and INPNC antisera (Figure 1). A band corresponding to the expected size of INPNC−OPH was detected from cells carrying pPNCO33, while no such protein was detected with *P. putida* JS444 carrying the parental plasmid pVLT33.

To assess the distribution of the fusion protein between the membrane and soluble fractions, total cell-free lysate, membrane, and soluble fractions were probed with anti-OPH sera. As shown in Figure 2, more than 98% of the fusion was associated with the membrane fraction as judged by the intensity of the protein band, which agrees well with the ratio of OPH activity between whole cells (8.56 U/mg protein) and cell lysates (8.73 U/mg protein). The whole cell OPH activity of the recombinant *P. putida* JS444 is on par with that observed with the recombinant *Moraxella* sp. (9.85 U/mg protein).

To investigate whether the INPNC−OPH fusion proteins were displayed correctly on the bacterial surface in a stable conformation, immunofluorescence microscopy was used. Cells were probed with the rabbit anti-OPH serum as a primary antibody and then fluorescently stained with an FITC-labeled goat anti-rabbit IgG antibody. As shown in Figure 3B, cells harboring pPNCO33 were brightly fluorescent, indicating that the INPNC−OPH fusion was successfully displayed on the surface. Cells carrying parental plasmid pVLT33 were not stained

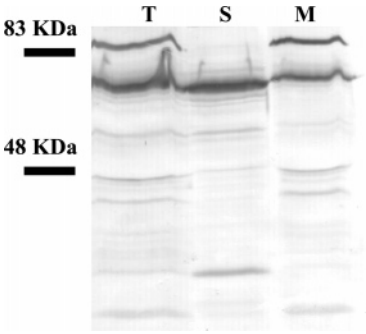

Figure 2. Distribution of INPNC−OPH fusion in different cellular fractions. The amount of INPNC−OPH in total cell lysate (T), membrane fraction (M), and soluble fraction (S) was probed by Western blot analysis. OPH antisera were used at a 1:3000 dilution.

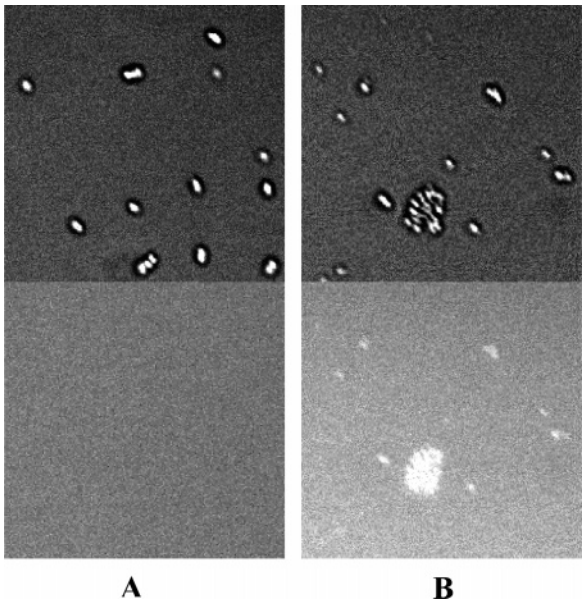

Figure 3. Immunofluorescence micrographs of *Pseudomonas putida* JS444 harboring (A) pVLT33 and (B) pPNCO33. Cells were probed with anti-OPH antisera and fluorescently stained with goat anti-rabbit IgG−FITC conjugate. The phase-contrast pictures are shown on top.

at all (Figure 3A). A similar result was also observed with the different *E. coli* strains (*20*) and *Moraxella* sp. (*14*).

Simultaneous Degradation of Organophosphates and PNP. One serious problem associated with engineered *Moraxella* sp. with surface-expressed OPH is the instability of bacteria behavior. In every 3 out of 4 cultivations, cells lost the ability to degrade either organophosphates or PNP. We hypothesized that high-level expression of OPH on the surface of *Moraxella* sp. results in membrane instability and significantly reduces either OPH activity or PNP degradation. This instability problem was, however, resolved with engineered *P. putida* JS444. This can be attributed to compatibility of the ice-nucleation protein (INP) anchor with the membrane structure of *P. putida* JS444 since INP was originally isolated from a similar species, *P. syringae* INA5.

To demonstrate the fast degradation of organophosphate pesticides and PNP by *P. putida* JS444 with surface-expressed OPH, cells were cultivated, harvested, and resuspended in the buffer for degradation of 0.4 mM paraoxon or PNP. As depicted in Figure 4A, paraoxon was very rapidly degraded within the first 42 min with almost stoichiometric release of PNP. Initial PNP degradation

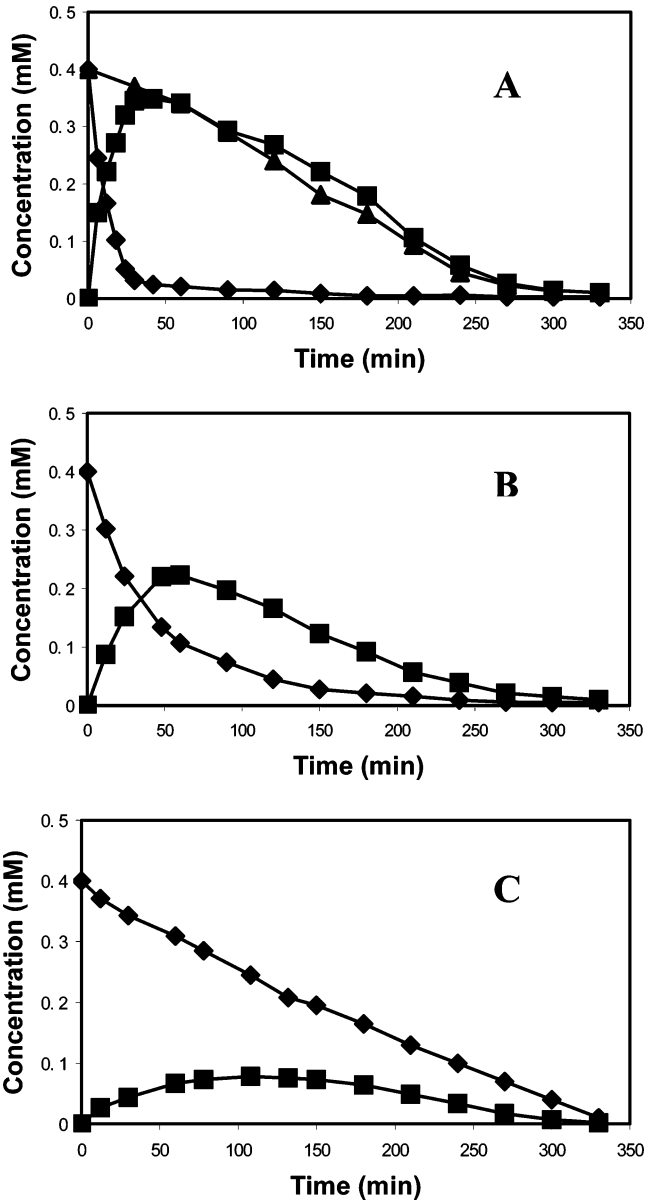

Figure 4. Simultaneous degradation of organophosphates and PNP by *Pseudomonas putida* JS444 harboring pPNCO33. (A) Paraoxon, (B) parathion, or (C) methyl parathion. All substrates were added at an initial concentration of 0.4 mM. Symbols: (◆) organophosphorus pesticides; (■) PNP formed during the hydrolysis of OPs; (▲) degradation of 0.4 mM PNP by *P. putida* JS444 harboring pPNCO33.

(1.29 μmol/h/mg dry weight) occurred on a slower time scale compared with the initial hydrolysis of paraoxon (7.90 μmol/h/mg dry weight). Both 0.4 mM PNP and paraoxon were completely mineralized in 5.5 h.

Similar degradation experiments were also performed to demonstrate that other pesticides could be similarly degraded. Complete hydrolysis of parathion (3.54 μmol/h/mg dry weight) and methyl parathion (1.53 μmol/h/mg dry weight) occurred within 150 and 330 min, respectively (Figure 4, B and C). This reduction in hydrolysis rate is consistent with the kinetic properties of OPH, which is more efficient in hydrolyzing paraoxon than other organophosphates (*26*). Again, the PNP released from hydrolysis was completely degraded within 5.5 h for parathion and methyl parathion, respectively.

Compared to the first reported use of recombinant *Moraxella* sp. (natural PNP degrader) with surface-expressed OPH (*14*), the complete mineralization of

organophosphates and PNP by the engineered *P. putida* JS444 strain is almost 2-fold faster. This rapid mineralization can be attributed to the faster PNP degradation rate by *P. putida* JS444 (1.29 μmol/h/mg dry weight) vs *Moraxella* sp. (0.6 μmol/h/mg dry weight) (*14*).

Conclusions

Various surface expression systems have been developed for Gram-negative bacteria, including *E. coli* (*19*, *20*, *22*−*24*), *Salmonella* sp. (*25*), *Pseudomonas* (*16*), and *Moraxella* sp. (*14*). In this paper, we demonstrated that OPH could be successfully targeted onto the surface of *P. putida* JS444, a natural PNP degrader, using the INP anchor. The resulting recombinant strain is capable of rapidly and simultaneously degrading organophosphate pesticides and PNP without instability problems associated with the engineered *Moraxella* sp. The recombinant *P. putida* JS444 also showed faster mineralization of PNP, paraoxon, methyl parathion, and parathion when compared to engineered *Moraxella* sp. (*14*). This advantage could contribute to better OPH activity and faster PNP degradation in the engineered *P. putida* JS444. These features, such as excellent stability of engineered *P. putida* JS444 and the improved degradation rate for OPs and PNP, make it an ideal biocatalyst for mineralization of organophosphates and PNP in bioreactor operations.

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