

Cell Surface Display of Organophosphorus Hydrolase in *Pseudomonas putida* Using an Ice-Nucleation Protein Anchor

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A surface anchor system derived from the ice-nucleation protein (INP) from *Pseudomonas syringae* was used to localize organophosphorus hydrolase (OPH) onto the surface of *Pseudomonas putida* KT2440. Cells harboring the shuttle vector pPNCO33 coding for the INP–OPH fusion were capable of targeting OPH onto the cell surface as demonstrated by whole cell ELISA. The whole cell activity of *P. putida* KT2440 was shown to be 10 times higher than those of previous efforts expressing the same fusion protein in *Escherichia coli*. The capability of expressing enzymes on the surface of a robust and environmentally benign *P. putida* KT2440 should open up new avenues for a wide range of applications such as *in situ* bioremediation.

Introduction

Contamination of soil, sediments, and groundwater with organophosphate (OP) compounds continues to be a problem in the world today. With advances in biotechnology, new efforts have been undertaken which emphasize the use of microorganisms for the degradation of pollutants rather than their disposal. Bioremediation is also attractive because it is far less disruptive and costly than conventional methods.

Naturally isolated soil microorganisms *Pseudomonas diminuta* MG and *Flavobacterium* sp. have been shown to express high activities of organophosphorus hydrolase (OPH) (1, 2), which hydrolyzes organophosphate pesticides. Hydrolysis of parathion, for example, reduces the toxicity by nearly 120-fold and leads to the formation of *p*-nitrophenol (3). Although the use of natural isolates as biocatalysts is an attractive strategy for treatment of OP compounds, the inaccessibility of the pesticides across the cell membrane reduces the overall catalytic efficiency.

Ice-nucleation protein (INP), an outer membrane protein from *Pseudomonas syringae*, is composed of three distinct domains: an N-terminal domain (15%) containing three or four potential transmembrane spans, a C-terminal domain (4%), and a central domain composed of repeating residues which acts as the template for ice nucleation (4). Both INP and truncated INP fusions have been constructed, successfully targeting various enzymes and antigens onto the surface of *Escherichia coli* (5–8) and *Salmonella* sp. (9). We have recently demonstrated that OPH can be displayed on the surface of *E. coli* (10) and *Moraxella* sp. (11) using the INP anchor. In both cases, over 70% of the INP–OPH fusions were targeted correctly to the outer membrane, resulting in greater access of the substrate and improved hydrolysis.

However, lab-born bacterial strains are not suitable for *in situ* remediation since they are not adapted to these environments. A more realistic approach is to engineer

soil bacteria that are known to survive in contaminated environments for an extended period. Of particular interest is *Pseudomonas putida* KT2440, which is a derivative of the TOL-pathway-containing *P. putida* mt-2 (12). This bacterium is robust and ubiquitous in soils and is capable of using a variety of pollutants such as toluene and xylene. They have been shown to express heterogeneous genes and to colonize a variety of soils (13, 14). *P. putida* KT2440 has also been classified as a nonpathogen by the National Institutes of Health. In addition, the complete genome has now been sequenced (15, 16). These desirable traits make this bacterium very attractive as a platform for *in situ* bioremediation. It has been genetically engineered for applications in bioremediation (17) and whole cell biocatalysis (18).

In this work, our goal is to target OPH onto the surface of *P. putida* KT2440. The result is a single bacterium that is endowed with the capability to rapidly degrade organophosphate pesticides and other pollutants. To date, this is the first report of targeting functional enzymes onto the surface of a *Pseudomonas* species, which should open up new avenues in treating recalcitrant environmental pollutants.

Experimental Protocol

Bacterial Strains and Plasmids. *P. putida* KT2440 was used in this study. The construction of plasmid pPNCO33 containing the INP–OPH fusion was described elsewhere (11). Strains bearing plasmids were grown in Luria–Bertani (LB) media supplemented with kanamycin to a final concentration of 50 mg/mL. Cells were grown in 250 mL flasks in an Innova 4000 shaker (New Brunswick Science) with vigorous agitation (300 rpm) at 30 °C. Bacteria harboring expression vectors were grown to an OD₆₀₀ = 0.4 before induction with 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside).

For transformation of plasmid, *P. putida* KT2440 was grown overnight in LB medium at 30 °C and 300 rpm. Overnight cultures were subcultured in 50 mL of LB medium to an OD = 0.4, harvested, and incubated in 5 mL of 0.1 M MgCl₂ for 2 h. Competent cells were stored in 30% glycerol at –80 °C until used. After thawing, 50 ng of pPNCO33 was added to 100 μ L of competent cells,

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and the mixture was incubated on ice for 1 h. After incubation at 42 °C for 2 min in a water bath, 200 μ L of LB medium was added, and the resulting mixture was incubated for an additional 2 h at 30 °C and 300 rpm. Finally, 100 μ L of cells was spread on LB agar plates containing 50 μ g/mL kanamycin. Transformants appeared after ~36–48 h at 30 °C.

Whole Cell OPH Assay. *P. putida* KT2440 cells harboring pPNCO33 were grown for either 24 or 48 h after IPTG induction, harvested, and resuspended in 50 mM citrate–phosphate buffer, pH 8.0, with 50 μ M CoCl₂. The enzyme activity was measured spectrophotometrically and conducted in a 1.5 mL disposable methacrylate cuvette (Fisher, Tustin, CA). The cell lysate was prepared by addition of 10 μ L 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 μ L of 1.0 OD₆₀₀ cells was added to 890 μ L of citrate–phosphate buffer and 100 μ L of 20 mM paraoxon (Sigma) in 10% methanol. Changes in absorbance (412 nm) were measured for 2 min at 37 °C. Activities were expressed in U (micromoles of paraoxon hydrolyzed per minute) per mg of protein ($e_{410} = 16500 \text{ M}^{-1} \text{ cm}^{-1}$ for *p*-nitrophenol). Similar whole cell activity measurements were conducted with *E. coli* XL1-Blue harboring pPNCO33.

Cell Fractionation. After 24 and 48 h of growth, cells were harvested and resuspended in 25 mM Tris–Cl buffer, pH 8.0. Cells were disrupted by a French pressure cell at 16000 psi (SLM Instruments, Inc.). Cells were passed through the French Press twice to ensure complete lysis. The crude extract was then centrifuged for 10 min at 11000g to remove any remaining cell debris. The cell-free extract was then centrifuged at 115000g (Beckman) to separate the membrane and soluble fractions. The supernatant representing the soluble fraction was retained, and the membrane fraction pellet was resuspended in the same volume of 25 mM Tris–Cl pH 8.0 buffer. A 10 μ L sample of total lysate, membrane, and soluble fractions was used for Western blot analysis.

Western Blot Analysis. Samples (10 μ L) of 2.5 \times concentrated cells were mixed with loading buffer, and the mixtures were boiled for 10 min. Samples were run on a 10% (w/v) acrylamide SDS–PAGE gel. Proteins were then transferred to a nitrocellulose support before incubation with either OPH (19) or INP (6) specific antibodies. Western blot analysis was performed using a Bio-Rad Immun-Blot GAR-AP kit (BioRad, Hercules, CA). Pre-stained broad-range molecular weight markers were used to estimate protein weights.

Whole Cell ELISA. Cells harboring the parental shuttle vector pVLT33 (20) and pPNCO33 were grown for 48 h after induction with 1 mM IPTG. After harvesting, cells were resuspended in 100 μ L of phosphate saline buffer (PSB) to a final OD₆₀₀ of 0.1 and immobilized onto a microtiter plate overnight at 4 °C. Excess cells were discarded, and the wells were then blocked with 2% BSA in PBS for 2 h. After being washed twice with PBS, the wells were incubated with anti-INP sera (1:1000) for 1 h, and probed with goat anti-rabbit antibody conjugated with alkaline phosphatase (1:3000). The wells were extensively washed and prepared for visualization. *p*-Nitrophenyl phosphate (Sigma) tablets were used as a substrate and dissolved (1 mg/mL) in 50 mM citrate–phosphate buffer, pH 8.0, and the absorbance in each well was monitored at 405 nm after 30 min.

Results and Discussion

To target OPH onto the surface of *P. putida* KT2440, a broad-host-range vector, pPNCO33, was used. Expres-

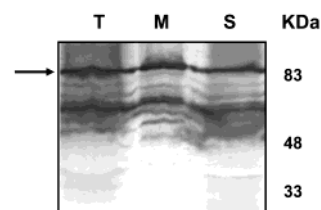


Figure 1. Western blot analysis of *P. putida* KT2440 harboring pPNCO33. Samples from total cell lysates (T), membrane fractions (M), and soluble fractions (S) were analyzed. OPH antibodies were used as a 1:5000 dilution.

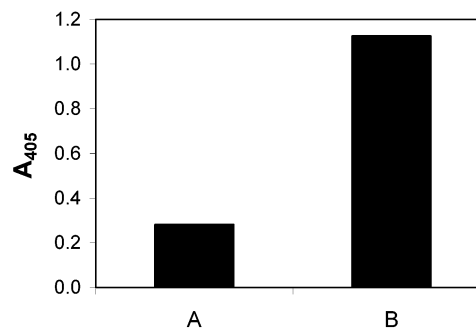


Figure 2. Whole cell ELISA of *P. putida* KT2440 harboring (A) the parental shuttle vector pVLT33 and (B) the surface expression vector pPNCO33. Polystyrene microplates were coated with cells at OD₆₀₀ = 0.1 and incubated with anti-INP sera, followed by a goat anti-rabbit antibody–alkaline phosphatase conjugate. Alkaline phosphatase activity was measured at 405 nm using *p*-nitrophenyl phosphate as the substrate.

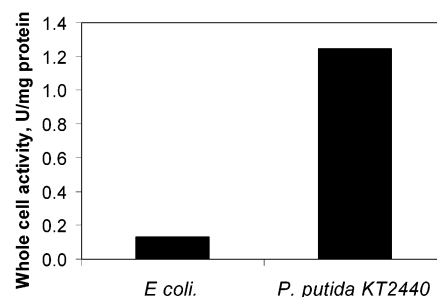


Figure 3. Whole cell OPH activities of *E. coli* XL-1 Blue and *P. putida* KT2440 harboring pPNCO33.

sion of the INPNC–OPH fusion was under the control of a *tac* promoter and induced with 1 mM IPTG. Induced cultures were viable and fully active during prolonged incubation for 48 h. To assess the distribution of the fusion protein between the membrane and soluble fractions, 48 h samples were harvested, lysed by a French Press, and fractionated into membrane and soluble fractions by ultracentrifugation. Total cell-free lysate (before ultracentrifugation), membrane, and soluble fractions were probed with anti-OPH sera. A major band of approximately 85 kDa, corresponding to INP–OPH fusion, was detected. Approximately 60% of the fusion was associated with the membrane fraction (Figure 1), consistent with expected surface localization of the OPH fusions. The activity distribution between the membrane and soluble fractions was similar to that of the Western blot analysis. Over 65% of the total activity was found in the membrane fraction, while the remaining 35% remained in the soluble fraction. Whole cell ELISA was used to ascertain the surface expression of the INP–OPH fusion. After incubation with an anti-OPH antibody, the recombinant strain harboring pPNCO33 showed a 5-fold higher intensity than the control *Pseudomonas* species

carrying the parental vector pVLT33 (Figure 2), indicating the expected surface localization of OPH.

To evaluate the efficiency of surface expression in *P. putida* KT2440, whole cell activities of cells harboring pPNCO33 were compared with that of *E. coli* XL-1 Blue carrying the same plasmid. As shown in Figure 3, whole cell activities were 10-fold higher for *P. putida* KT2440 in comparison to *E. coli*. This significant increase in surface targeting of OPH in *Pseudomonas* KT2440 is likely a result of improved translocation since the INP anchor is derived from a related *Pseudomonas* species. Similar to results from previous studies (10, 11, 19), maximum whole cell activity was obtained after 48 h of incubation. The whole cell activity of 1.446 U/mg of protein after 48 h was almost 10 times higher than the value of 0.142 U/mg of protein at 24 h. Since the expression level of the INP-OPH fusions remained constant between the 24 and 48 h cultures (data not shown), these results again suggest that stationary-phase translocation or processing of previously synthesized OPH fusions is responsible for the improved whole cell activity. In addition to paraoxon, whole cells with OPH on the surface degraded other pesticides such as parathion and methylparathion (data not shown).

Conclusion

Various surface expression systems have been developed for Gram-negative bacteria, including *E. coli* (10, 21–24), *Salmonella* sp. (9) and *Moraxella* sp. (11). However, there are no reports of the surface expression of functional enzymes on the surface of *Pseudomonas* sp. Using the INP surface anchor, OPH was successfully targeted onto the cell surface, and the recombinant *Pseudomonas* species showed 10-fold greater catalytic activity compared to *E. coli*. The feasibility to display a wide range of functional enzymes on the surface of *P. putida* KT2440 offers a strong platform toward the successful utilization of this environmentally robust bacterium for a wide range of biocatalysis applications.

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