

Cell Surface Display of Organophosphorus Hydrolase Using Ice Nucleation Protein

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A new anchor system based on the ice nucleation protein (InaV) from *Pseudomonas syringae* INA5 was developed for cell surface display of functional organophosphorus hydrolase (OPH). The activity and stability of cells expressing the truncated InaV (INPNC)-OPH fusions were compared to cells with surface-expressed OPH using two other fusion anchors based on Lpp-OmpA and the truncated InaK protein. Whole cell activity was as much as 5-fold higher using the InaV anchor. Majority of the OPH activity was located on the cell surface as determined by protease accessibility and cell fractionation experiments. The surface localization of OPH was further verified by immunofluorescence microscopy. Constitutive expression of OPH on the surface using the InaV anchor resulted in no cell lysis or growth inhibition, in contrast to the Lpp-OmpA anchor. Suspended cultures also exhibited good stability, retaining almost 100% activity over a period of 3 weeks. Therefore, the InaV anchor system offers an attractive alternative to the currently available surface anchors, providing high-level expression and superior stability.

Introduction

As the demand for agricultural produce increases, so inevitably does the need for pesticides. Currently organophosphorus compounds are one of the most widely used pesticides in industrialized countries. In the United States, over 10 million kg of organophosphate pesticides are applied annually (1). High-level exposure to organophosphates results in acetylcholine (ACh) accumulation, which interferes with muscular responses and in vital organs produces serious symptoms and eventually death. Repeated or prolonged exposure to organophosphates can cause delayed cholinergic toxicity and neurotoxicity (2). Current techniques for detoxifying organophosphate pesticides rely on harsh chemical treatment, incineration, and landfills. Chemical methods present waste disposal problems because of the large volumes of acid and base used in detoxification. Landfills pose a threat of leaching and, ultimately, groundwater contamination. Incineration invokes public opposition due to potential toxic emissions release into the atmosphere (3). Because of the environmental concern associated with the accumulation of these pesticides in food products and water supplies, there is a great need to develop safe, convenient, and economically feasible methods for pesticide detoxification.

Enzymatic degradation of organophosphorus nerve agents has been a subject of considerable attention during the past 10 years. Organophosphorus hydrolase (OPH) isolated from soil microorganisms has been shown to degrade organophosphates effectively (4, 5). However, practical applications of large-scale enzymatic degradation have always been limited by the cost and stability of OPH. As a cost-effective alternative, whole cells (either

growing or nongrowing) rather than enzymes, can be immobilized onto supports (such as in an immobilized-cell bioreactor). However, the mass transport limitations of substrates and products across the cell membrane raise a new problem due to the ability of the outer membrane to act as a permeability barrier that prevents substrates from interacting with the enzymes residing within the cell (6). This bottleneck, however, could be eliminated if OPHs are displayed onto the surface of cells.

Recently, OPH was functionally expressed onto the cell surface of *E. coli* using an Lpp-OmpA(46–159) fusion system (7). Cultures with surface-expressed OPH degraded parathion and paraoxon effectively without the transport limitation observed in cells expressing OPH intracellularly. However, constitutive expression of Lpp-OmpA-OPH resulted in severe growth inhibition. Cells in liquid cultures tended to aggregate and were prone to cell lysis. Long-term storage on plate or in frozen stock resulted in a significant reduction in OPH activity. Although this instability problem was resolved when a tightly regulated, inducible promoter was used to express OPH on the cell surface, the use of an inducer is not economical for large-scale application. Ideally, high-level expression of OPH on the cell surface could be sustained without induction.

The ice-nucleation protein (INP), an outer membrane protein from *Pseudomonas syringae*, is capable of catalyzing the formation of ice in supercooled water (8). INP is composed of three distinct domains: a N-terminal domain (15%) containing three or four potential transmembrane spans, a C-terminal domain (4%), and a central domain composed of repeats given by a 8-, 16- and 48-residue periodicity that acts as template for ice nucleation (9–12). Recently, INP has been used as an anchor for the cell surface expression of several enzymes and proteins (13–18). Even the truncated version of INP containing only the N- and C-terminal portion (INPNC)

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was shown to be sufficient for targeting proteins to the cell surface (13). In this work, we demonstrated the functional expression of OPH onto the surface of *E. coli* using the INPNC anchor. The resulting cultures showed high levels of OPH activity without induction; the growth inhibition and cell lysis problems associated with the Lpp-OmpA anchor were no longer detected. Owing to their high activity and stability, these "live biocatalysts" are ideal for large-scale detoxification of organophosphorus pesticides.

Materials and Methods

Bacterial Strains and Plasmids. *Escherichia coli* strains XL1-Blue (*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* (r_k^- , m_k^+), *supE44*, *relA1*, *lac* [F' , *proAB*, *lacI^qZDM15*, Tn10 (Tet^r)] and JM105 (*endA1*, *thi*, *rpsL*, *sbcB15*, *hsdR4*, Δ (*lac-proAB*), [F' , *traD36*, *proAB*, *lacI^qZDM15*]) were used in this study. Plasmids pDP245 (19) and pJHC1 (13) were used as the templates for the *inaV* and *inaK* genes, respectively. Plasmid pJK33 (5) was used for the intracellular expression of OPH. Plasmid pCOPH-C1 (20) carrying a hybrid *cbd-opd* gene was used as the source of the *opd* gene. Plasmid pOP131 was used for constitutive expression of OPH on the cell surface using the Lpp-OmpA anchor (7).

Strains bearing plasmids were grown in Luria-Bertani (LB) media supplemented with ampicillin to a final concentration of 100 μ g/mL. Cells were grown in 250-mL flasks in a Innova 4000 shaker (New Brunswick Sci.) with vigorous aeration (300 rpm) at either 25, 30, or 37 °C.

Construction of Ice-Nucleation OPH Fusion. The truncated InaV-OPH fusion was constructed as follows. PCR was used to amplify the N- and C-terminal domains using plasmid pDP245 as the template. Primers 5'-GGGAATTCAGGAAACAATGAATATCGACAAAGCGTTGGTA -3' and 5'-CCGGTACCAATCAGATCACTGTG-GTTGCCAGC-3' were used to amplify the N-terminal region, and primers 5'-CCGGATCCTTCTCGACCTC-TATCCAGTC-3' and 5'-CCGGTACCGGCGGGCATG-GCTAGCAGC-3' were used for the C-terminal region. The resulting 500 bp N-terminal fragment was digested with *EcoRI* and *KpnI* and ligated into a similarly digested pUC18, resulting in pIVN. The 300 bp C-terminal domain was *KpnI/BamHI* digested and ligated into pIVN to give pIVNC. To create an in frame INPNC-OPH fusion, a 1.1 kb fragment of the *opd* gene was *BamHI* and *HindIII* digested from pCOPH-C1 and inserted into similarly digested pIVNC to generate pINCOP. Plasmid pIKNCOP containing the truncated InaK-OPH fusion was constructed similarly except a different forward primer (5'-GGGAATTCAGGAAACAATGAATCTCCGACAAGGC-3') was used for the N-terminal portion to account for the differences in sequence between the two *ina* genes. Plasmid pJHC1 containing the complete *inaK* gene was used as the template for PCR.

Organophosphorus Hydrolase Assay. Cells were grown in LB broth at 30 °C and harvested after 2 days of incubation. Cells were centrifuged and resuspended in 50 mM citrate-phosphate buffer with 50 μ M CoCl₂, pH 8.0. Activity assays were conducted in 1.5-mL disposable methacrylate cuvettes (Fisher, Tustin). For each assay, 200 μ L of cells (OD₆₀₀ = 1.0) were added to 700 μ L of citrate-phosphate buffer and 100 μ L of 20 mM paraoxon (Sigma) in 10% methanol. Change in absorbance (412 nm) was measured with a Beckman spectrophotometer for 2 min at 37 °C. Activities are expressed as μ mol of paraoxon hydrolyzed per min (U) per OD₆₀₀ whole cells (ϵ_{410} = 16,500 M⁻¹ cm⁻¹ for *p*-nitrophenol).

Proteinase Accessibility Assay. Cells harboring pINCOP, pIKNCOP, and pJK33 were centrifuged and resuspended in 1 mL of 15% sucrose, 15 mM Tris-HCl, 0.1 mM EDTA, pH 7.8. Samples were incubated for 1 h with 5 μ L of 20 mg/mL proteinase-K at room temperature. To inhibit further proteinase K activity, 10 μ M of phenylmethylsulfonylfluoride was added after incubation. Proteinase K treated and untreated cells were assayed for OPH activity as described above.

Cell Fractionation. Cells grown in LB medium at 30 °C were harvested after 2 days incubation and centrifuged at 5000 rpm for 10 min (Beckman). Cells were resuspended in 10 mL of 25 mM Tris-HCl, pH 8.0. Lysozyme (100 μ g/mL) was added, and the cells were incubated for 1 h on ice. Cells were disrupted by a French pressure cell at 16,000 psi (SLM instruments, Inc.). After two passes through the French press, cells were centrifuged for 10 min at 10,000 rpm to remove cell debris. The supernatant was ultracentrifuged at 115,000 $\times g$ to separate the membrane and soluble portions. The supernatant was collected as the soluble fraction, while the pellet representing the membrane fraction was resuspended in the same 25 mM Tris-HCl buffer.

Immunofluorescence Microscopy. After 2 days of growth at 30 °C, cells were centrifuged and resuspended (0.5 OD₆₀₀) in phosphate-buffered saline (PBS) with 3% BSA. Cells were incubated for 8 h at 4 °C with rabbit anti-OPH IgG (1:3000) (20). The cells were washed extensively and resuspended in PBS with goat anti-rabbit IgG conjugated with FITC (Sigma) at a dilution of 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 (Nikon).

Stability Study of Resting Cultures. Cultures of JM105(pOP132) and JM105(pINCOP) were grown in 50 mL of LB medium for 2 days, washed twice with 50 mL of 150 mM NaCl solution, resuspended in 5 mL of 50 mM Na₂HPO₄-citric acid buffer solution (pH 8.0) containing 50 μ M CoCl₂, and incubated in a shaker at 37 °C. Over the 3-week duration, 0.1 mL of samples were removed each day. Samples were centrifuged and resuspended in 0.1 mL of 50 mM citrate-phosphate buffer with 50 μ M CoCl₂, pH 8.0. OPH assays were conducted as described above.

Results

Surface Expression of OPH Using Ice Nucleation Protein. Among the different *Pseudomonas* species capable of ice nucleation, there exists a heterogeneous selection of ice-nucleation proteins, InaK, InaZ and InaV, each differing slightly from one another. So far, the targeting of proteins and enzymes to the cell surface has been demonstrated only with the InaK protein from *P. syringae* KCTC1832 (13–18) as the anchor. This anchor system targeted proteins to the surface in both *E. coli* and *Salmonella* at high efficiency with no apparent decrease in cell viability or compromise in membrane integrity (14, 18). We were interested in whether other INPs could be similarly employed for the same purpose. The InaV protein from *Pseudomonas syringae* INA5 was chosen in this study because it is the most structurally diverse from the previously used InaK system. There is only 77% sequence homology between the two proteins. Most of the differences occur at the N-terminal domain, which is thought to be the region responsible for targeting INP to the outer membrane of the cell (9).

The truncated version of INP (INPNC) was used to generate fusions with OPH since INPNC showed com-

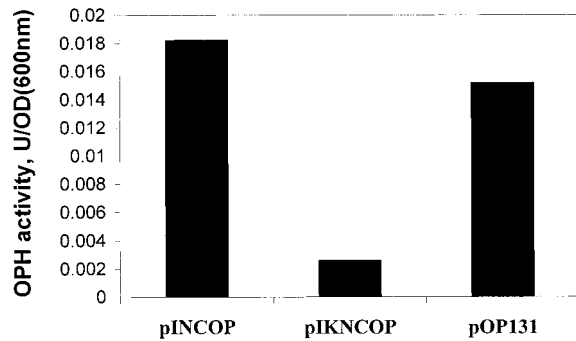


Figure 1. Whole cell activity of *E. coli* XL1-Blue expressing OPH on the surface using either the InaV (pINCOP), InaK (pIKNCOP), or Lpp-OmpA (pOP131) anchor. Cells were grown under identical conditions as described in Materials and Methods.

parable stability and no decrease in activity from the full-length INP (13). Both truncated fusion constructs (InaK and InaV) involved the use of only the specific N- and C-terminal ends of the INP excluding the native internal repeats. The truncated INPNC fragments for both the *inaK* and *inaV* derived genes were generated by fusing two PCR fragments, one from the N-terminal end of the INP gene and the other from the C-terminal end. The resulting truncated fragments were fused with the *opd* gene to generate pIKNCOP (InaK) and pINCOP (InaV), respectively.

To explore whether the differences in the N-terminal region between InaK and InaV would lead to improved stability and expression of OPH on the cell surface, we compared the efficiency of targeting OPH on the cell surface using the two different INP anchors. To demonstrate the functional expression of OPH, recombinant cells were grown at 30 °C and whole cell activity was measured. As shown in Figure 1, cells carrying pIKNCOP showed 5-fold lower activity than cells carrying pINCOP, while whole cell activity of XL1-Blue(pINCOP) was similar to that of cells carrying pOP131 using the Lpp-OmpA anchor. Incubation at either 25 or 37 °C resulted in lower activity for both INP anchors (data not shown). Although OPH activity was successfully achieved with either INP anchors, these results demonstrate that the InaV anchor may be more efficient in targeting proteins to the cell surface. As a result of the lack of activity of the truncated fusion based on InaK, only the properties of the newly constructed InaV-based system were assessed.

Surface Localization of OPH. The percentage of OPH on the cell surface was estimated by measuring OPH activity in the membrane and soluble cell fractions. Over 70% of the activity was detected in the membrane fractions (Table 1). In parallel, more than 70% of OPH activity was present on the cell surface as judging from the activity ratio between whole cells and cell lysates (Table 1). Finally, protease accessibility experiments were performed to ascertain the surface localization of OPH. Cells were incubated with proteinase K in the presence of EDTA. Since proteinase K cannot readily diffuse across the cell membrane, degradation should only occur with proteins exposed on the surface. After 1 h of incubation, the OPH activity for cells carrying pINCOP decreased by 64%, while cells expressing OPH intracellularly (pJK33) had only a 7% drop in activity. All of these results collectively suggested that the majority of INPNC-OPH fusion proteins were present on the cell surface.

To clearly demonstrate the surface localization of OPH, immunofluorescence microscopy was used. Cells were

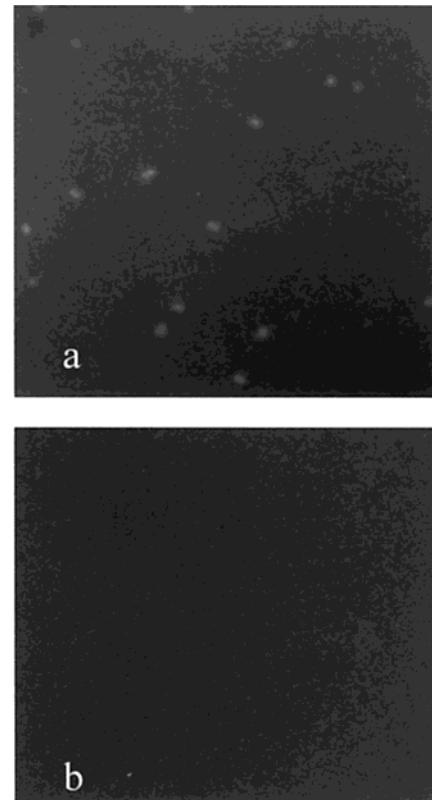


Figure 2. Immunofluorescence micrographs of *E. coli* XL1-Blue harboring (a) pINCOP and (b) pUC18. Cells were probed with anti-OPH antisera and fluorescently stained with goat anti-rabbit IgG-FITC conjugate.

Table 1. Percentage of Surface-Exposed OPH As Estimated from Proteinase K Treatment, Whole Cell Versus Lysate Assays, and Membrane Fractionation Experiments; Experiments Conducted with *E. coli* XL1-Blue Carrying Either pINCOP or pJK33

plasmid	% decrease in activity in proteinase K treated cells	whole cell activity (as % of lysate activity)	% activity in membrane fraction
pINCOP	64	71	73
pJK33	7	1	5

probed with rabbit anti-OPH serum as a primary antibody and then fluorescently stained with FITC-labeled goat anti-rabbit IgG antibody. As shown in Figure 2a, cells harboring pINCOP were brightly fluorescent, indicating that OPH was successfully displayed on the surface. Control cells carrying only pUC18 were not stained at all with the FITC-labeled secondary antibody (Figure 2b). From all of these results, we concluded that OPH was indeed displayed functionally on the cell surface using the INPNC anchor.

Stability of Cultures Expressing INPNC-OPH Fusions. One of the major problems associated with constitutive expression of OPH on the surface using the Lpp-OmpA system is the instability observed in term of cell viability and OPH activity. A new anchor system not only has to be stable but also target proteins in large quantities to the cell surface.

To test whether constitutive expression of INPNC-OPH inhibits cell growth, growth kinetics of cells carrying pINCOP or pUC18 were compared. No growth inhibition was observed for cells expressing INPNC-OPH. Both cultures reached the same final cell density after 48 h of incubation (Figure 3). Because constitutive expression of outer membrane protein can result in membrane desta-

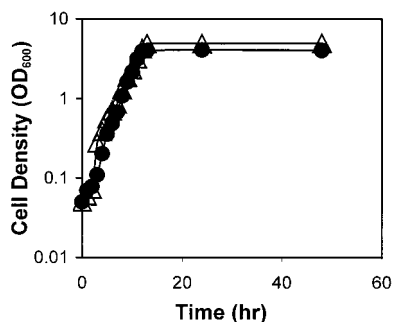


Figure 3. Cell growth kinetics of *E. coli* XL-1 Blue harboring pUC18 (Δ) and pINCOP (●).

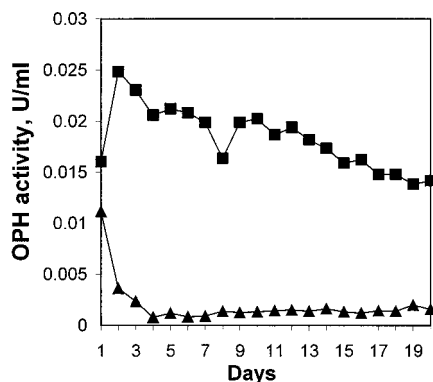


Figure 4. OPH activity in suspended *E. coli* cultures expressing OPH constitutively on the surface with either the Lpp-OmpA (▲) or the InaV (■) anchor. Samples (0.5 mL) were taken each day and assayed for OPH activity.

bilization and cell lysis, the stability of suspended cultures was monitored; whole cell activity was determined periodically over a 3-week period. As shown in Figure 4, whole cell activity of JM105(pOP131) continued to drop over the 3-week period, while that of JM105(pINCOP) remained at essentially the original level. The stability of the cells observed here is in line with previous reports using the INP system for the surface expression of other proteins (13–14).

Discussion

Surface expression offers improved biodegradation of the desired compound and becomes increasingly important when more water insoluble substrates such as PCBs and PAHs are used. The Lpp-OmpA anchor system provides very efficient transport of OPH onto the cell surface, thereby overcoming the diffusional limitations of substrate uptake. One drawback in using the Lpp-OmpA anchor is that it causes membrane instability leading to cell lysis. Although this can be resolved by tightly regulating expression, an alternative solution is to employ another surface anchor that provides stable expression without induction. The INP-based anchor system, which has been used previously for the surface-expression of carboxymethylcellulase (13) and luevansucrase (14), was explored for this possible. Two truncated INP anchors, one based on the previously described InaK protein and one based on a similar InaV protein, were used. Surprisingly, OPH fusions based on InaK resulted in poor surface localization when compared to Lpp-OmpA or InaV. Even growth at 25 °C, a condition reported to be optimal for the InaK anchor (18), failed to improve the whole cell activity. Although functionally similar, the InaK and InaV proteins share only 77% homology. Much of the diversity occurs in the critical N-terminal region, which interacts with the phospholipid moiety of the outer

membrane (9, 21). The increased expression level of the InaV-based anchor system may be the result of improved interaction with the outer membrane. More importantly, the InaV anchor offers superior stability, showing no sign of cell lysis or growth inhibition. Resting cultures maintained close to 100% activity after 3-week incubations.

In conclusion, the newly explored InaV-based fusion system reported in this paper offers an attractive alternative for surface-expression of proteins or enzymes. The InaV anchor provides higher level of surface expression as well as superior stability. Functional expression can be maintained without the use of a tightly regulated promoter. This system may be ideal for the development of whole cell degradation of organophosphates.

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