J. Microbiol. Biotechnol. (2012), **22**(2), 234–238
http://dx.doi.org/10.4014/jmb.1104.04011
First published online October 21, 2011

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Surface Display of Organophosphorus Hydrolase on *E. coli* Using N-Terminal Domain of Ice Nucleation Protein InaV

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Received: April 11, 2011 / Revised: October 10, 2011 / Accepted: October 14, 2011

Recombinant *Escherichia coli* displaying organophosphorus hydrolase (OPH) was used to overcome the diffusion barrier limitation of organophosphorus pesticides. A new anchor system derived from the N-terminal domain of ice-nucleation protein from *Pseudomonas syringae* InaV (InaV-N) was used to display OPH onto the surface. The designed sequence was cloned in the vector pET-28a(+) and then was expressed in *E. coli*. Tracing of the expression location of the recombinant protein using SDS–PAGE showed the presentation of OPH by InaV-N on the outer membrane, and the ability of recombinant *E. coli* to utilize diazinon as the sole source of energy, without growth inhibition, indicated its significant activity. The location of OPH was detected by comparing the activity of the outer membrane fraction with the inner membrane and cytoplasm fractions. Studies revealed that recombinant *E. coli* can degrade 50% of 2 mM chlorpyrifos in 2 min. It can be concluded that InaV-N can be used efficiently to display foreign functional protein, and these results highlight the high potential of an engineered bacterium to be used in bioremediation of pesticide-contaminated sources in the environment.

Keywords: Ice nucleation protein, organophosphorus hydrolase, surface display, diazinon, chlorpyrifos

Organophosphate is a general name for phosphoric acid esters and is the basis of many pesticides and herbicides. Organophosphorus pesticides (OPs) are one of the most common chemicals used for protecting agricultural products and livestock, and cover approximately 34% of the common insecticides used throughout the world [1]. The toxicity of such compounds is attributed to their ability to react with acetylcholinesterase, so that they perturb the cholinesterase activity and imitate the enzyme's substrate [8]. To remove these chemicals from the environment, such methods as chemical treatment, landfills, or incineration were used; however, these methods were reported as being problematic owing to poison exposure and economical limitations [3, 12]. Biological procedures for degradation of OPs have attracted growing interest in recent years [4]. The enzyme degrading the organophosphorus compounds was first identified in *Flavobacterium* ATCC 27551 and *Pseudomonas diminuta* and was named phosphotriesterase (PTE). The genes coding this enzyme were similar and all located on bacterial extrachromosomal plasmid [6]. PTE is a 72 kDa homodimer with a high catalytic efficiency within a short time interval [5]. The high costs of enzyme purification and decrease of its stability limit its practical application in large scales. As a cost-effective alternative, the whole cell can be used instead of the enzyme; however, in this method, the organophosphorus compound should transmit through the outer membrane [15]. To overcome the transmitting problem and increase the detoxification rate of organophosphorus compounds, different anchoring motifs with the ability to cross the plasma membrane and outer membrane have been used to target the organophosphorus hydrolase (OPH) onto the cell surface, including the Lpp-OmpA chimer, ice nucleation protein (INP), and auto-transporters [10, 13–15, 18]. INP is a protein in the outer membrane of *Pseudomonas syringae*, which is able to ice catalyze at subfreezing temperatures (–2°C to –4°C) [2]. It is composed of N-terminal (15%), C-terminal (4%), and central (81%) domains [7, 15]. InaK, InaV, and InaZ are different types of ice nucleation proteins, which have the signal peptide sequence and the outer membrane anchoring system [15]. The amino domain of InaK was used to display OPH and green fluorescent protein (GFP) and results demonstrated that this domain alone is sufficient to present the enzyme to the cell surface [7, 12]. In a study by Xu *et al*. [16], the amino domain of InaV was used to display GFP and the results showed that InaV-N is able to

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present the foreign protein. In the present study, OPH was presented to the cell membrane using the InaV-N domain; the amino domain of INP of *Pseudomonas syringae* strain Ina5 is used for the first time to present the OPH to the surface of *E. coli*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media Conditions

E. coli BL21 DE3 PlysS was used as the host cell of the pET-28a(+)-*inaVN-opd* plasmid. Cells harboring the recombinant plasmid were grown in 15 ml of LB media with 50 mg/l kanamycin at 150 rpm, 37°C. Cells harboring pET-28a(+)-*inaVN-opd* plasmids were induced with 1 mM IPTG and cultured at 37°C for 3 h.

Construction of Ice-Nucleation-OPH Fusion and Its Transformation

The truncated *inaVN-opd* fusion was constructed as follows. The sequence encoding the N-terminal domain of the *inaV* gene joined to the amino domain of the *opd* gene from *P. diminuta* (GenBank Accession No. M20392) was synthesized. *Nco*I and *Xho*I restriction sites were applied in the N-terminal and C-terminal domains of the sequence, respectively. The synthesized sequence was *Nco*I and *Xho*I digested and inserted into similarly digested pET-28a(+) to generate the pET-28a(+)-*inaVN-opd* plasmid. The plasmid was then transformed into *E. coli* BL21 DE3 PlysS using a (Bio-Rad) micro-pulser in 2000 V and in a 0.2 cm cuvette.

Medium for the Growth of Recombinant Cells

Chlorpyrifos (Cp) and diazinon (Di) with 99.5% purity were purchased from Sigma. The 20 mM solution of toxins was made in 50% acetonitrile. Mineral salts medium (MSM) containing diazinon was prepared to evaluate the ability of the recombinant strain to utilize the toxic chemical. It contained 0.1 g/l NaCl, 0.2 g/l KCl, 0.5 g/l $(NH_4)_2SO_4$, 50 mg/l $CaCl_2{\cdot}H_2O$, 0.2 g/l $MgSO_4{\cdot}7H_2O$, and 20 mg/l $MgSO_4{\cdot}7H_2O$. Then, 50 ml of MSM was prepared in a 250 ml Erlenmeyer flask supplemented with 1,500 mg/l diazinon as the sole source of carbon and phosphorus. Afterwards, the flasks were inoculated with 200 µl of the recombinant strain (after 3 h induction), *P. aeruginosa* IRLM1 (native strain containing OPs-degrading enzyme, unpublished data), and *E. coli* BL21, separately. The MSM containing diazinon and *P. aeruginosa* IRLM1 was used as the positive control and that containing diazinon and *E. coli* BL21 as the negative control. In order to investigate the ability of the recombinant strain to degrade diazinon, after 7 days the samples were cultured from Erlenmeyer flasks to MSM agar plates supplemented with 50 mg/l diazinon.

Cell Fractionation

The method proposed by Li *et al*. [11] was used to trace the expression site of OPH. Cells harboring *inaVN-opd* hybrids were harvested and resuspended in PBS buffer containing 1 mM EDTA and lysozyme at 10 µg/ml to set as unit cell density (OD_{600}=1) and incubated for 2 h at room temperature. The cell suspension was treated with an ultrasound sonication at 30 s × 2cycles. To obtain a total membrane fraction, the whole cell lysate was pelleted by centrifugation at 14,000 rpm for 2 h using an ultracentrifuge. For further outer membrane fractionation, the pellet (total membrane fraction) was resuspended with PBS buffer containing 0.01 mM $MgCl_2$ and 2% Triton X-100 and was incubated for 30 min at room temperature for solubilizing the inner membrane (IM), and then the outer membrane (OM) fraction was repelleted after 2 h centrifugation at 14,000 rpm. The isolated components were used in the next stages. To investigate the site of OPH expression, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) analysis of components of cells harboring pET-28a(+)-*ina*V-N was applied. Cytoplasm and membrane fractions of *P. aeruginosa* IRLM1 were prepared for further analysis as described below.

Assessment and Tracing of the OPH Activity

Cellular activity was assessed based on a method by Yang *et al.* [17]. Cell fractions were used in this study. The outer membrane fraction of cells expressing InaVN-OPH and total membrane of strain IRLM1 were both resuspended in PBS buffer (pH=7.4) to set as unit cell density; separated IM and cytoplasm fractions were prepared. Enzyme activity was studied by following the decrease in optical density of Cp using a UV/VIS spectrophotometer (in a disposable methacrylate cuvette) at 215 nm (in 2 and 3 min). For each assay, 10 µl of each fraction was added to 890 µl of PBS buffer and 100 µl of 20 mM Cp in 50% acetonitrile. Enzyme tracing was studied by removing 0.5 ml of prepared samples (samples that were exposed to Cp after 3 min incubation) and transferring to 2 ml HPLC vials and analyzed by a HPLC (Cecil 1100). The HPLC condition was as follows: a Zorbax SB-C18 column (250 × 4.6 mm^2, 5 Rm) used at ambient temperature; the mobile phase consisting of acetonitrile (80%), distilled water (19.5%), and acetic acid (0.5%), with a flow rate of 1.5 ml/min. Changes in absorbance (215 nm) were measured.

Stability Study of InaVN-OPH-Expressing Cells

To investigate the stability of InaVN-OPH-expressing cells, expression in cells harboring the pET-28a(+)-*inaVN-opd* plasmid and those containing pET-28a(+) were induced for 3 h. Then, 20 µl of each sample was inoculated to 5 ml of LB medium containing 50 mg/l kanamycin. To evaluate whether expression of outer membrane protein results in membrane destabilization and cell lysis, the optical density of the suspended cultures was monitored by UV/VIS spectrophotometer at 600 nm.

RESULTS AND DISCUSSIONS

Growth of InaVN-OPH-Expressing Cells in MSM

The results showed that the cells harboring the pET-28a(+)-*inaVN-opd* plasmid could grow in a medium containing diazinon as the only source of carbon and phosphorus. No growth in the negative control was observed. These results indicate that the recombinant strain, as like *P. aeruginosa* IRLM1, degrades the organophosphorus compound of diazinon and utilizes it as the source of energy (Fig. 1). The growth of the samples was detected quantitatively too, using a UV/VIS spectrophotometer at 600 nm, and the results showed the rapid growth of the recombinant strain in comparison with the native one, indicating that not only the expression of the active OPH is mediated by this

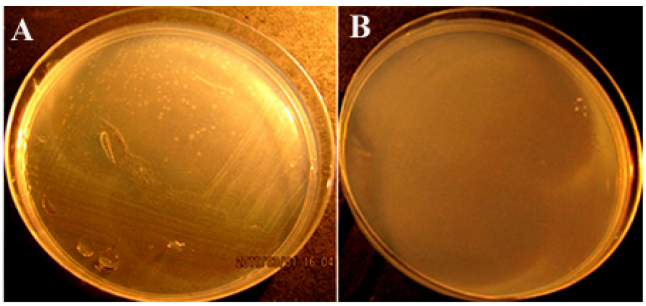

Fig. 1. Ability of the recombinant strain to degrade diazinon and to utilize it as a sole source of energy.
(**A**) Recombinant strain displaying OPH could grow in MSM agar supplemented with Cp. (**B**) *E. coli* BL21 lacking OPH activity could not grow in MSM agar supplemented with Cp as the negative control.

system, but also that the recombinant strain overcomes the limiting step of the process (*i.e.*, the cell membrane).

Location of OPH in InaVN-OPH-Expressing Cells

A Gram-negative bacterium is composed of inner membrane, outer membrane, and cytoplasm. To investigate the site of OPH expression in components of the recombinant cells, SDS–PAGE was applied (Fig. 2). Lane 1 represents the outer membrane, Lane 3 is the inner membrane, and Lane 4 is the cytoplasm. The 58.9 kDa band related to the recombinant protein InaVN-OPH was only observed in the

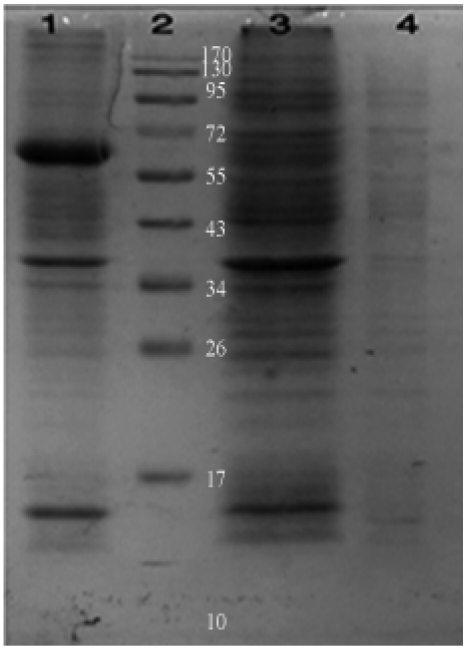

Fig. 2. Expression of InaVN-OPH in different fractions of the induced cell.
Lane 1: The outer membrane, which shows the band of about 58.9 kDa (between 55 kDa and 72 kDa) related to InaVN-OPH; Lane 2: Protein marker Sm0333; Lane 3: The inner membrane, which lacks the band of InaVN-OPH; Lane 4: Cytoplasm, which lacks the band of InaVN-OPH.

column of outer membrane. Thus, it is concluded that OPH is fully presented on the cell surface. These results corroborate the study by Kong *et al*. [7] and reveal that the amino domain of the *ina* gene alone is able to present the protein to the surface of the host cell.

Furthermore, these results confirm a study by Xu *et al*. [16] that reported the analysis of the total expression level and surface localization of N-GFP, NC-GFP, and N-GFP-C and demonstrated that the truncated InaV from *P. syringae* INA5 could be used to display foreign protein in *V. anguillarum*. This is the first report of the ability of the N-terminal domain of InaV to express OPH on the surface of the bacterial membrane.

Stability of InaVN-OPH-Expressing Cells

One of problems encountered in surface expression of OPH using the Lpp-OmpA system is the instability of the host cell, leading to decrease of the cell viability and enzyme activity. This can be overcome by changing the promoter adjusting this system [17]. To investigate whether the InaVN anchor influences cellular stability, we compared the growth of InaVN-OPH-expressing cells with those containing pET-28a(+) (Fig. 3). The growing scheme of *E. coli* BL21 corresponded to that of the InaVN-OPH-expressing cells and both reached their final concentration after 48 h. This suggests that OPH expression on the bacterial surface using the InaVN anchor does not inhibit the cellular growth.

Assay of Displayed OPH Activity

In this study, the ability of the whole cell and its components to degrade Cp was evaluated based on changes in the optical density of Cp (Table 1). When the cell lacking the *opd* gene (the negative control) was exposed to Cp, no change was observed in the optical density of Cp and absorbance

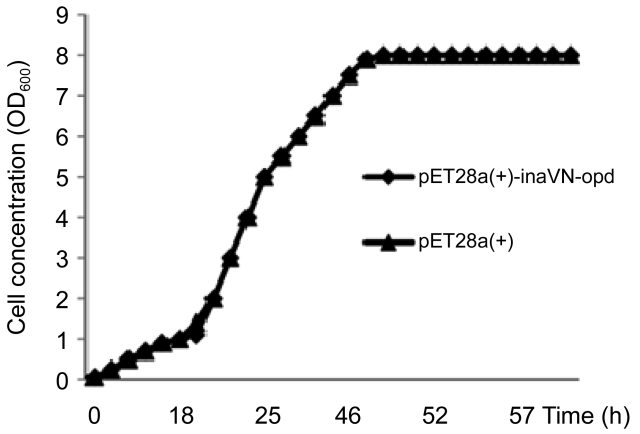

Fig. 3. Growth comparison of the recombinant strain with *E. coli*.
Cells were cultured in LB medium and cell density was monitored.
(◆) Cells harboring pET-28a(+)-*inaVN-opd* plasmid. (▲) Cells harboring pET-28a(+) plasmid.

Table 1. Activity of the enzyme expressed on the surface.

Bacteria	OD^{a}_{215} T=0	OD_{215} T=2	OD_{215} T=3
Negative Control	~[b]1.1	~1.1	~1.1
P. aeruginosa IRLM1 total membrane	~1.1	~1	~1
P. aeruginosa IRLM1 cytoplasm	~1.1	0.537	0.323
OM of cells harboring pET-28a(+)-*ina*VN-opd	~1.1	0.573	0.376
IM of cells harboring pET-28a(+)-*ina*VN-opd	~1.1	~1.1	~1.1
Cytoplasm of cells harboring pET-28a(+)-*ina*VN-opd	~1.1	~1	~1

[a]Optical density of Cp at 215 nm.
[b]Tilde symbol (~) implies an estimation.

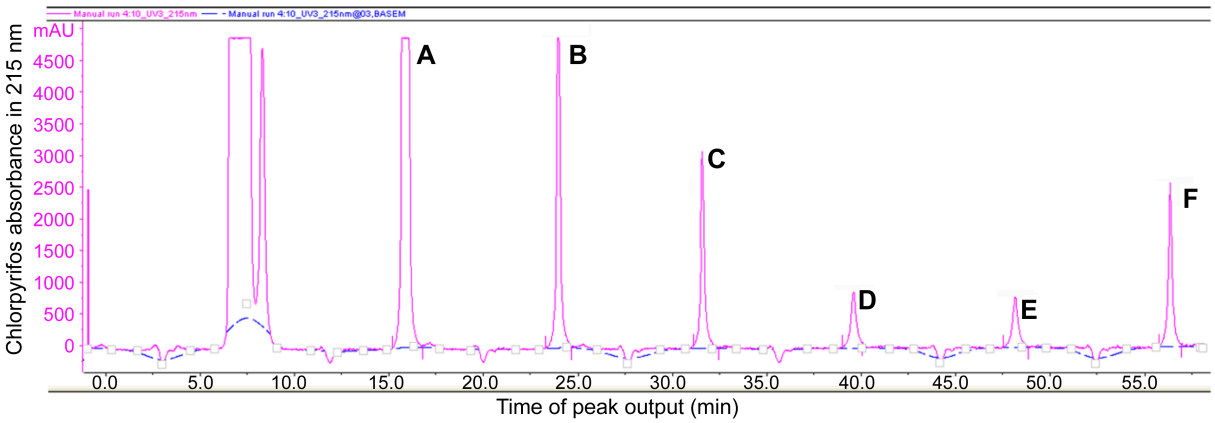

Fig. 4. Comparison of cell fractions activity after 3 min incubation of samples in 2 mM Cp.
Each peak represents the amount of hydrophobic chlorpyrifos that comes out of the C_{18} column at indicated retention time. (**A**) Cells without *opd* gene as negative control. (**B**) IM fraction of cells harboring pET-28a(+)-*inaVN-opd*. (**C**) Cytoplasm fraction of cells harboring pET-28a(+)-*inaVN-opd*. (**D**) OM fraction of cells harboring pET-28a(+)-*inaVN-opd*. (**E**) Cytoplasm fraction of IRLM1 strain. (**F**) Membrane fraction of IRLM1 strain. HPLC analysis shows that cell activity of recombinants locates in the OM fraction.

remained constant (approximately 1.1) after 2 and 3 min. When the cytoplasm of native strain *P. aeruginosa* IRLM1 (the positive control that internally expresses OPH) was exposed to Cp, the optical density of Cp decreased after 2 and 3 min. As shown, when the outer membrane of cells harboring pET-28a(+)-*inaVN-opd* was exposed to Cp, the absorbance of Cp decreased, whereas the inner membrane and cytoplasm of cells harboring pET-28a(+)-*inaVN-opd* showed no changes in Cp optical density.

These results revealed that cells harboring pET-28a(+)-*inaVN-opd* can express OPH on the cell surface, and therefore in the presence of such strains, the Cp is directly exposed to the enzyme and is degraded. Study of enzyme activity showed that cells harboring the pET-28a(+)-*inaVN-opd* plasmid can degrade 50% of 2 mM Cp in 2 min.

Study of Chlorpyrifos Degradation Using HPLC

The location of the OPs-degrading enzyme was detected by comparing enzyme activity in whole cell with cell fractions (Fig. 4). Data of HPLC analysis are shown (Table 2). The area under the peak represents the quantitative analysis of Cp by HPLC. The OPs-degrading activity of the total membrane and cytoplasm fractions of *P. aeruginosa* IRLM1 are shown as controls. In comparison with the total membrane fraction of the IRLM1 strain, the cytoplasm fraction showed enzymatic activity, whereas the outer membrane fraction of cells harboring pET-28a(+)-*inaVN-opd* showed the enzymatic activity and revealed that OPH is sufficiently surface-displayed.

Table 2. Data of HPLC analysis.

No.	Retention (min)	Peak start (min)	Peak end (min)	Area[a] (mAu*min)
1	15.75	15.12	16.73	2,729.3936
2	23.92	23.24	24.64	1,345.0478
3	31.57	31.08	32.41	661.7491
4	39.58	38.96	40.00	323.0647
5	48.1	47.49	48.82	307.5119
6	56.32	55.71	56.91	627.9202

[a]Represents the area under the peak indicating the amount of Cp.

These results confirm that the amino domain of the InaV anchor is appropriate for displaying the protein, and unlike the amino domain of the InaK anchor, the presented protein in this method is highly active, and unlike the Lpp-OmpA anchor, it does not inhibit growth. The recombinant strain expresses the active enzyme on the surface and overcomes the rate-limitation, and therefore it can be a highly efficient method for biodegradation of organophosphorus compounds.

Acknowledgment

The authors would like to thank all colleagues in the Applied Biotechnology Research Center of Baqiyatallah Medical Sciences University, Analytical Chemistry and Biology departments, for their kind contribution in the research.

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