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Comparison of the Organophosphorus Hydrolase Surface Display Using InaVN and Lpp-OmpA Systems in *Escherichia coli*

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The purpose of this study was to compare the ability of an engineered *Escherichia coli* to degrade chlorpyrifos (Cp) using an organophosphorus hydrolase enzyme, encoded in both *Flavobacterium* sp. ATCC 27551 or *Pseudomonas diminuta*, by employing the Lpp-OmpA chimera and the N-terminal domain of the ice nucleation protein as anchoring motifs. Tracing of the expression location of the recombinant protein using SDS-PAGE showed the presentation of OPH by both anchors on the outer membrane. This is the first report on the presentation of OPH on the cell surface by Lpp-OmpA under the control of the *T7* promoter. The results showed cell growth in the presence of Cp as the sole source of energy, without growth inhibition, and with higher whole-cell activity for both cells harboring plasmids pENVO and pELMO, at approximately 10,342.85 and 10,857.14 U/mg, respectively. Noticeably, the protein displayed by pELMO was lower than the protein displayed by pENVO. It can be concluded that Lpp-OmpA can display less protein, but more functional OPH protein. These results highlight the high potential, of both engineered bacteria, for use in the bioremediation of pesticide-contaminated sources in the environment.

Keywords: Lpp-OmpA, InaVN, organophosphorus hydrolase, cell surface display, whole-cell activity, chlorpyrifos

Introduction

The toxic action of organophosphorus pesticides (OPs) and chemical warfare agents is achieved by two major toxic effects; the first is a covalent binding within the active site of acetylcholinesterase (AChe), perturbing cholinesterase activity and imitating the enzyme's substrate. The second effect is organophosphate-induced delayed polyneuropathy, associated with the inhibition of at least 70% neuropathy target esterase activity [7, 9]. Serious contamination issues arise at waste disposal sites close to agricultural fields and at OP production facilities, due to the large-scale manufacture and inappropriate handling of the organophosphate, which leads to contamination of the soil and air, and both surface and groundwater [11, 17]. Organophosphate is the common name of phosphoric acid esters, and most organophosphorus pesticides have a similar general structure containing three phosphoester linkages, and can be divided into oxon OP (P=O) and thion OP (P=S) groups. In general, hydrolysis of one of the phosphoester bonds reduces the toxicity of an OP [2, 5, 6]. Organophosphorus hydrolase (OPH) is a zinc-containing homodimeric protein identified in *Flavobacterium* sp. ATCC 27551 and *Pseudomonas diminuta*, isolated from soils in the Philippines and the United States, respectively, and has been named phosphotriesterase (PTE) [5, 24]. The *opd* (organophosphorus-degrading) gene in *Flavobacterium* sp. ATCC 27551 and in *Pseudomonas diminuta* is identical; however, they are located in two different plasmids [3]. Several organophosphate-degrading enzymes have been isolated and characterized; however, the degradation rate is very low owing to a mass transfer issue, which reduces the overall catalytic efficiency [21, 23]. The transport problems across the outer cell membrane can be overcome by an alternative strategy, which is the use of displayed OPH by surface-displaying anchors, including Lpp-OmpA chimer, ice nucleation protein (INP), and auto-transporters [13, 15, 19, 22, 26]. The Lpp-OmpA chimera consists of a signal sequence and the first nine N-terminal amino acids

Table 1. Primers used in fragment constructs.

Primer	Primer sequence	Underlined restriction site
P1	TTGGATCCATGAAA GCTACTAAACTGGTAC	*Bam*HI
P2	AGGAATTCCTGATCGATTTTAGCGTT GCTG	*Eco*RI
P3	TTGAATTCAACCCGTATGTTGGCTTT	*Eco*RI
P4	AAGAGCTCGTTGTCC GGAGCAGTG	*Sac*I
P5	AAGAGCTCCAAACGAGAAGGGTT GT	*Sac*I
P6	TACTCGAGTCAGTGAAT GAAGGCCATC	*Xho*I

of major *E. coli* lipoprotein (Lpp) joined to a transmembrane domain (amino acids 46–159) from outer membrane protein A (OmpA). The efficiency of the Lpp-OmpA hybrid was the first successful approach and is well known to direct polypeptides to the cell surface, such as ß-lactamase, cellulases, the scFv antibody, the cellulose-binding domain, cyclodextrin glucanotransferase, and the chitin-binding domain, on the surface of *E. coli* [18, 25]. We previously developed a new anchor system to display OPH [8]. In this paper, for the first time we have used the Lpp-OmpA chimera for the functional display of OPH under promoter *T7* control and also compared its activity and surface targeting efficiencies with InaVN-OPH.

Materials and Methods

Vectors and Bacterial Strains Media Conditions

S. dysenteriae, provided from the Milad Hospital, was used as the template for the *lpp* and *ompA* genes. *E. coli* BL21(DE3) plysS was used as a host cell for recombinant plasmids. Plasmid pET-28a(+) (Novagen, USA) was used for expressing the Lpp-OmpA and the OPH fusion hybrid. pET-28a(+)-*inaVN-opd* (pENVO) [8] was used for expressing truncated InaV and the OPH fusion hybrid and as a template of the *opd* gene. *P. aeruginosa* IRLM.1 was also used as the cytosolic expression control [12]. Cells harboring the recombinant plasmids were grown at 37°C in 15 ml of LB medium with 40 mg/l kanamycin at 150 rpm. Cells harboring recombinant plasmids were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, USA) after cells had grown to an OD_{600} = 0.6 and then incubated overnight at 37°C.

Table 2. Optical density of Cp over time.

Sample	OD^{a}_{215} T = 0	OD_{215} T = 2	OD_{215} T = 3
Non-induced E. coli BL21(DE3) pLys	~ 1.1[b]	~ 1.1	~ 1.1
P. aeruginosa IRLM1 total membrane	~ 1.1	~ 1.1	~ 1
P. aeruginosa IRLM1 cytoplasm	~ 1.1	~ 0.537	~ 0.323
OM of cells harboring pELMO	~ 1.1	~ 0.519	~ 0.340
IM of cells harboring pELMO	~ 1.1	~ 1.1	~ 1.1
Cytoplasm of cells harboring pELMO	~ 1.1	~ 1.1	~ 1.1
OM of cells harboring pENVO	~ 1.1	~ 0.573	~ 0.376
IM of cells harboring pENVO	~ 1.1	~ 1.1	~ 1.1
Cytoplasm of cells harboring pENVO	~ 1.1	~ 1.1	~ 1.1

[a]Optical density of Cp at 215 nm.

[b]Tilde symbol (~) implies an estimation.

Construction of *lpp-ompA-opd* Fusion and Transformation

Primers are described in Table 1 and PCR products are shown in Table 2.

lpp and *ompA* fragments were amplified from *S. dysenteriae* using primers P1/P2 and P3/P4, respectively. The *opd* fragment was amplified from plasmid pENVO using P5/P6. The amplified products were analyzed by electrophoresis with a 1% agarose gel containing SYBR Green Stain and visualized by Gel Doc XR documentation (Bio-Rad, USA). Both *lpp* and *ompA* products were *Eco*RI digested and ligated using T4 DNA ligase (Fermentas, Germany) at 22°C for 3 h. The resultant product was used as a template to amplify the *lpp-ompA* fragment using P1/P4. Both *lpp-ompA* and *opd* fragments were *Sac*I digested and ligated in the same conditions as has been described above, and the ligation product was used to generate the *lpp-ompA-opd* fragment by primers P1/P6. Produced *lpp-ompA-opd* was *Bam*HI and *Xho*I digested and inserted into similarly digested pET-28a(+) to generate the plasmid pELMO. After each digestion, the fragments were purified with a GeneJET Gel Extraction Kit (Fermentas). Plasmid pET-28a(+) isolation was carried out using a GeneJET

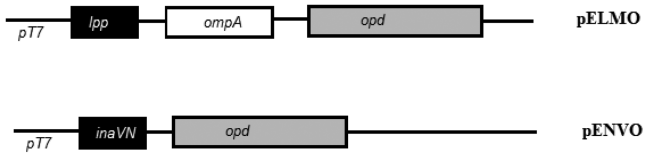

Fig. 1. Gene maps of recombinant plasmids harboring truncated *inaVN/opd* and *lpp-ompA-opd* fusions.
Plasmid pET-28a (+) was used as a parent vector for constructing these fusions.

Plasmid Miniprep Kit (Fermentas) as per the manufacturer's protocol. *E. coli* BL21(DE3) pLys was transformed with the plasmids pELMO and pENVO (Fig. 1) using a micro-pulser (Bio-Rad, USA) in a 0.1 cm electroporation cuvette [4].

Tracing of Lpp-OmpA-OPH and inaVN-OPH Proteins

Recombinant cells were grown at 37°C in 5 ml of LB medium with 40 mg/l kanamycin at 150 rpm, and then were induced with 0.1 mM IPTG and incubated for 5 h at 37°C. To trace the Lpp-OmpA-OPH and the InaVN-OPH proteins, the induced cells were harvested and fractionated according to the method of Li *et al* [14]. Harvested cells were resuspended in PBS buffer containing 1 mM EDTA and 10 µg/ml lysozyme, set as a unit cell density of 1 (OD_{600} nm), and incubated for 2 h at room temperature. The cell suspension was treated with an ultrasound sonication at 30 sec × 2 cycles. To obtain the total membrane fraction, whole-cell lysate was pelleted by centrifugation at 18,000 ×*g* 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, thus solubilizing the inner membrane, and then the outer membrane fraction was repelleted after 2 h centrifugation at 18,000 ×*g*. The isolated components were then used in the next stages. To investigate the expression site of OPH, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 12% running gel using the method of Laemmli [10] and protein bands were visualized by staining with Coomassie Brilliant Blue R250 (Merck, Germany). The cytoplasm and membrane fractions of *P. aeruginosa* IRLM.1, as the cytosolic expression control, were also prepared for further analysis.

Culture Conditions for the Growth of Recombinant Cells

In order to study the ability of recombinant cells to degrade organophosphorus pesticides, the induced recombinant cells were harvested and inoculated on mineral salt medium (MSM) agar (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 $MgSO_4{\cdot}7H_2O$) supplemented with 50 µg/ml kanamycin and 50 mg/l Cp (99.5% purity) (Sigma-Aldrich) and incubated overnight at 37°C [12]. *P. aeruginosa* IRLM1 (native strain) and non-induced cells (negative control) were inoculated separately.

Stability Study of InaVN-OPH-Expressing Cells

To investigate the stability of OPH-displaying cells, expression was induced in cells harboring pENVO and pELMO plasmids and those containing pET-28a(+) 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 the outer membrane protein results in membrane destabilization and cell lysis, the optical density of the suspended cultures was monitored by a UV/VIS spectrophotometer at 600 nm.

Assay of OPH Activity

Prepared cell fractions were used to assay the enzyme activity by following the decrease in absorbance of Cp by a UV/VIS spectrophotometer (in disposable methacrylate cuvette) at OD_{215}. The outer membrane fractions were resuspended in PBS buffer to set the unit cell density (OD_{600} = 1). For each assay, 10 µl of each fraction was added to 890 µl of PBS buffer. Reactions were initiated with the addition of 2 mM Cp (dissolved in 60% acetonitrile) (Merck) and incubated for 2 and 3 min at 37°C [25]. Activities were expressed as µmol of Cp hydrolyzed per min (U) per OD_{600} whole cells (ε_{215} = 7 M^{-1} cm^{-1} for Cp).

Results and Discussion

Construction of Recombinant pELMO

The restriction enzyme digests were flanked by a 1 kb ladder (SM0333; Fermentas). The molecular sizes of the observed restriction fragments indicated the successful cloning of the *lpp-ompA-opd* fusion (Fig. 2).

Tracing of Lpp-OmpA-OPH and InaVN-OPH Proteins

The cell wall of gram-negative bacteria is composed of

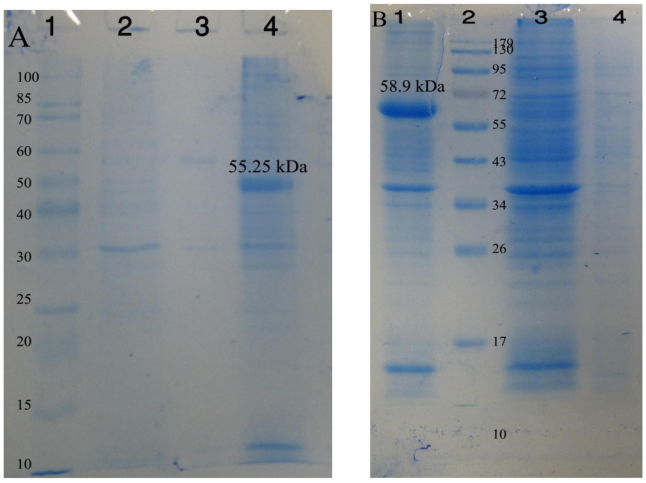

Fig. 2. Expression of OPH in different fractions.
(**A**) Cell fractions harboring pELMO. Lane **1**: Protein marker Sm0661; Lane **2**: The inner membrane, which lacks the band of Lpp-OmpA-OPH; Lane **3**: Cytoplasm, which lacks the band of Lpp-OmpA-OPH; Lane **4**: The outer membrane, which shows the band about 55.25 kDa (between 50 kDa and 60 kDa) related to Lpp-OmpA-OPH; Lane **5**: Cytoplasm, which lacks the band of Lpp-OmpA-OPH. (**B**) Cell fractions harboring pENVO. Lane **1**: The outer membrane, which shows a band 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.

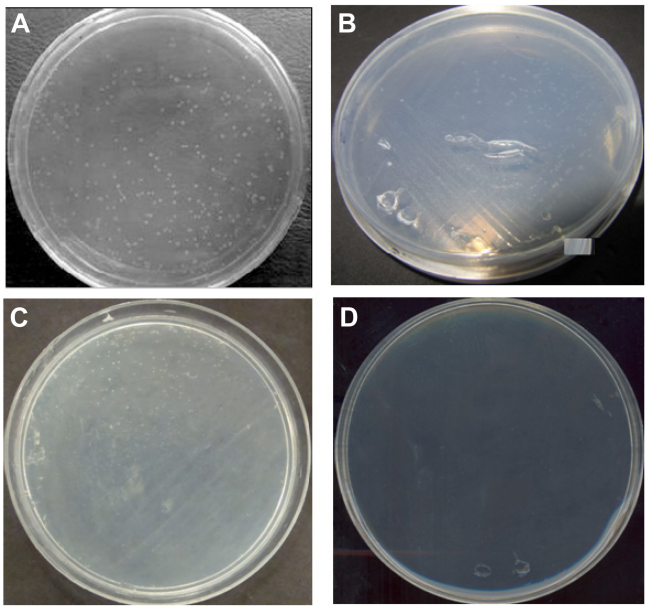

Fig. 3. The ability of the recombinant strain to degrade Cp and to utilize it as a sole source of energy.
(**A**) Recombinant strain harboring pELMO (expressing Lpp-OmpA-OPH). (**B**) Recombinant strain harboring pENVO (expressing InaVN-OPH). (**C**) *P. aeruginosa* IRLM1 (cytosolic expression control). (**D**) Non-induced cells as the negative control.

the inner membrane, outer membrane, and cytoplasm. To investigate the location of the OPH enzyme, *E. coli* BL21(DE3)pLys cells harboring the recombinant plasmids pELMO and pENVO that encode InaVN-OPH (cell surface displayed OPH using N-terminal INP) and Lpp-OmpA-OPH (cell surface displayed OPH using ompA), respectively, were grown in LB medium overnight under 0.1 mM IPTG induction. The Lpp-OmpA-OPH and the InaVN-OPH chimeric proteins were estimated to be a 55.25 kDa and 58.9 kDa protein, respectively. Analysis of the cell fractions harboring pELMO and pENVO in SDS-PAGE showed a new protein band related to the recombinant protein, only in the column of the outer membrane (Fig. 2). No protein band related to the desired proteins was detected in non-induced cells. As shown in Fig. 2, the protein band obtained using InaVN is thicker than the Lpp-OmpA-OPH protein band. Thus, it can be concluded that although OPH is fully presented on the cell surface using both anchors, under identical conditions the protein displayed by the InaVN anchor is significantly more than the protein displayed using the Lpp-OmpA anchor.

Growth of Recombinant Cells in MSM Agar

Growth of cells harboring pELMO and pENVO plasmids after IPTG induction and *P. aeruginosa* IRLM1 (cytosolic expression control) on MSM agar supplemented with 50 mg/l Cp as the only source of carbon and phosphorus is shown in Fig. 3. No growth was observed in the native control. These findings indicate that OPH can be functionally expressed using both anchors.

It is noteworthy to say that the primary metabolite 3,5,6-trichloro-2-pyridinol (TCP) is produced by the degradation of Cp, which has antimicrobial activity. If TCP is not degraded, it accumulates and suppresses microbial growth. In this study, bacterial growth observed in recombinants was achieved with low concentrations of Cp (50 mg/l), and no further growth was observed over time. Other studies have also shown that high concentrations of Cp result in smaller colonies. These findings indicate that TCP is naturally produced and limits bacterial growth.

Stability of Cultures Displaying Fusion Protein

As we discussed in our previous study [8], the growth scheme of *E. coli* BL21(DE3)pLys corresponds to that of the InaVN-OPH-expressing cells. Therefore, it was concluded that the InaVN anchoring system exhibits no growth inhibition.

The Lpp-OmpA system was first used to display OPH on the cell surface of *E. coli*. The findings of a previous study showed a successful display of active OPH on the outer membrane by the Lpp-OmpA hybrid, but membrane destabilization and growth inhibition were observed, which led to a decrease in cell viability and enzyme activity [18].

In another study, the methyl parathion hydrolase enzyme (MPH) was displayed by an Lpp-OmpA system with no change in permeability or cell growth. The problem was overcome by changing the promoter, which adjusted this system [25].

To study whether the designed pELMO plasmid inhibits cell growth, the growth kinetics of cells carrying pELMO and pENVO were compared. No significant growth inhibition was observed for cells carrying either plasmid. Both cultures reached the same final cell density as control samples after 48h of incubation (Fig. 4).

These results are due to the fact that in the case of promoter leakiness, a decrease in the viability of cells is observed. When the OPH fusion protein was controlled by an *lpp-lac* promoter that lacks a Cap site and is weakly constitutively active without IPTG induction, either cell growth inhibition or cell viability reduction was observed. Applying a tightly regulated *tac* promoter to display Lpp-OmpA-MPH-GFP fusions in *E. coli* results in no growth inhibition [25]. Consequently, the lack of growth inhibition

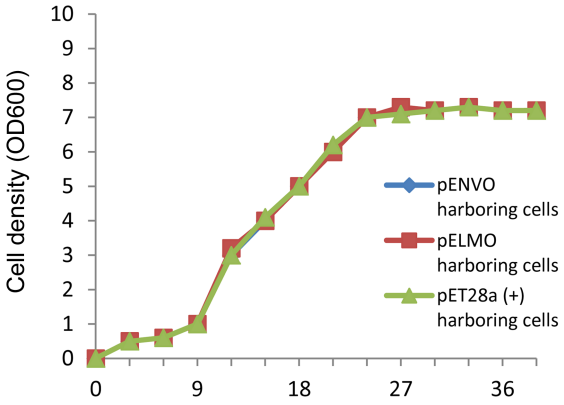

Fig. 4. Growth comparison of the recombinant strains with non-induced *cells*.

Cells were cultured in LB medium and the cell density was monitored after IPTG induction. (▲) Cells harboring pET-28a plasmid. (◆) Cells harboring pENVO plasmid. (■) Cells harboring pELMO plasmid.

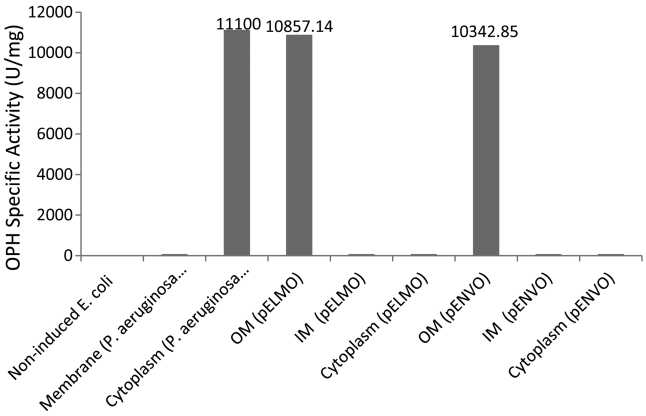

Fig. 5. The specific activity of different fractions.

The activity of cells displaying OPH proteins are only detected in the outer membrane fraction. The activity of native *P. aeruginosa* IRLM1 is only observed in the cytoplasm fraction. Non-induced cells are used as the control.

and cell instability in this study resulted in improved whole-cell activity. These findings are due to the *T7* promoter properties. When *T7* promoter is used, T7 RNA polymerase is inserted under control of a *lac* operator that is induced in the presence of IPTG. In conclusion, all the *T7* RNA polymerases of the cells just detect the *T7* promoter that only synthesizes RNAs with the *T7* promoter, and therefore, promoter leakiness is not observed. In the case of the *lac* promoter, however, gene synthesis is dependent on the RNA polymerase of the host cell, which is involved in other places [1, 16]. These results suggest the controllable expression of Lpp-OmpA-OPH by the *T7* promoter in response to IPTG and the efficiency to direct OPH to the outer membrane.

Assay of Displayed OPH Activity

Spectroscopy analysis evaluated the ability of the surface-displayed OPH to cleave the P-O bond. The decrease in Cp observed with fractions expressing OPH, compared with the relatively constant Cp in the presence of non-induced cells after 3 and 5 min, indicates the fact that induced cells can express and display functional OPH. The data of spectroscopy analysis are illustrated in Table 2. The activity assay of cells expressing InaVN-OPH and Lpp-OmpA-OPH in the degradation of Cp (P-O bond) using spectroscopy data showed a specific activity of 10,857.14 and 10,342.85 U/mg, respectively, only in the outer membrane fraction. The native strain showed a specific activity of 11,100 U/mg in the cytoplasm fraction (Fig. 5).

These results have shown that the surface display of OPH using both Lpp-OmpA and InaVN anchoring systems can increase the whole-cell OPH activity by reducing the mass transfer limitation of the substrate.

According to the results, Lpp-OmpA can display less protein but more functional OPH protein. In one study, it was reported that the whole-cell activity of *E. coli* XL1-Blue expressing OPH on the surface using either the InaV (pINCOP) or Lpp-OmpA (pOP131) anchors had significant functionality after two days of incubation. This result was observed while the whole-cell activity of cells harboring pOP131 (expressing Lpp-OmpA anchor) started to drop after the first day of inoculation [20]. The more functional OPH by Lpp-OmpA anchoring is probably due to a more sufficient folding of OPH in the Lpp-OmpA system, which can be caused by a difference in the membrane spans between both anchors. Moreover, a more accurate orientation of the enzyme active site might be created by the Lpp-OmpA domain, which leads to a better exposure to the substrate. Therefore, the low activity of whole cells displaying InaVN-OPH, despite high-levels of expression, is theorized as being due to the low substrate availability caused by the non-desired orientation of the OPH enzyme. Therefore, the Lpp-OmpA system looks more promising since it is well-suited as a carrier of relatively large inserts.

In conclusion, in the present study, an Lpp-OmpA anchor was employed under the control of *T7* promoter to present the OPH protein to the cellular surface. The evaluation of the Lpp-OmpA-OPH and InaVN-OPH expressing

strains to degrade Cp showed that both recombinant strains can grow in MSM agar with 50 mg/l Cp, whereas *E. coli* BL21(DE3) pLys containing pET28a(+) cannot grow in the minimal medium with toxin, does not degrade it, and has the same enzyme activity. These results confirm the fact that applying Lpp-OmpA, under *T7* promoter control, and InaVN as an anchor, both can lead to a functional enzyme display and it could be a highly efficient method for the biodegradation of organophosphorus compounds.

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