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Cotranslocation of Methyl Parathion Hydrolase to the Periplasm and of Organophosphorus Hydrolase to the Cell Surface of *Escherichia coli* by the Tat Pathway and Ice Nucleation Protein Display System▽

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A genetically engineered *Escherichia coli* strain coexpressing organophosphorus hydrolase (OPH) and methyl parathion hydrolase (MPH) was constructed for the first time by cotransforming two compatible plasmids. Since these two enzymes have different substrate specificities, the coexpression strain showed a broader substrate range than strains expressing either one of the hydrolases. To reduce the mass transport limitation of organophosphates (OPs) across the cell membrane, MPH and OPH were simultaneously translocated to the periplasm and cell surface of *E. coli*, respectively, by employing the twin-arginine translocation (Tat) pathway and ice nucleation protein (INP) display system. The resulting recombinant strain showed sixfold-higher whole-cell activity than the control strain expressing cytosolic OP hydrolases. The correct localization of MPH and OPH was demonstrated by cell fractionation, immunoblotting, and enzyme activity assays. No growth inhibition was observed for the recombinant *E. coli* strain, and suspended cultures retained almost 100% of the activity over a period of 2 weeks. Owing to its high level of activity and superior stability, the recombinant *E. coli* strain could be employed as a whole-cell biocatalyst for detoxification of OPs. This strategy of utilizing dual translocation pathways should open up new avenues for cotranslocating multiple functional moieties to different extracytosolic compartments of a bacterial cell.

Synthetic organophosphates (OPs) are widely used to control agricultural pests and account for ~38% of the total pesticides used globally (30). In the United States alone, over 40 million kg of OP pesticides are used annually (29). OPs are potent acetylcholinesterase (AChE) inhibitors, which leads to delayed cholinergic toxicity and neurotoxicity. OP poisoning is a worldwide health problem, and there are around 3 million poisonings and 200,000 deaths annually (31).

Organophosphorus hydrolase (OPH) is a zinc-containing homodimeric protein, and identical plasmid-borne *opd* genes coding for OPH have been found in two parathion-degrading bacteria, *Pseudomonas diminuta* MG and *Flavobacterium* sp. strain ATCC 27551 (23, 28). Hydrolysis of OPs by OPH reduces their toxicity by several orders of magnitude, providing a promising enzymatic detoxification technology (31). The rates of hydrolysis of some OP pesticides vary dramatically. For example, methyl parathion and chlorpyrifos are hydrolyzed by OPH 30- and 1,200-fold slower than the preferred substrate, paraoxon (6, 7). Cui et al. (8) described a chromosome-based *mpd* gene from methyl parathion-degrading *Plesiomonas* sp. strain M6 isolated in China. The *mpd* gene shows only 12% identity to *opd* at the amino acid level, and it encodes methyl parathion hydrolase (MPH), which has high catalytic activity with dimethyl OPs, suggesting that there is significant novelty in the gene-enzyme system.

Practical applications of large-scale enzymatic degradation have always been limited by the cost of purification and the stability of OPH. Although the use of whole cells as biocatalysts is an alternative strategy for treatment of OP pesticides, the very low permeability of the cell membrane for the pesticides dramatically reduces the overall catalytic efficiency (24). To overcome this substrate transport barrier, various strategies to enhance whole-cell biocatalytic efficiency have been developed, including treatment of cells with permeabilizing agents, surface display of OPH by an Lpp-OmpA or ice nucleation protein (INP) anchoring motif, and periplasmic secretion of OPH by a Sec or Tat pathway (15, 16, 24, 29).

INP is an outer membrane protein from *Pseudomonas syringae* that accelerates ice crystal formation in supercooled water. INP has a multidomain organization that includes an N-terminal domain containing three or four transmembrane spans, a C-terminal domain, and a highly repetitive central domain for ice nucleation (17, 35). Truncated INP derivatives containing N- and C-terminal domains or an N-terminal domain can serve as anchoring motifs for cell surface display of foreign proteins (20, 29).

The Tat pathway is a protein-targeting system dedicated to the export of folded proteins across the cytoplasmic membrane of bacteria (19, 33). Proteins exported by the Tat pathway are synthesized as precursors with N-terminal twin-arginine signal peptides having the consensus motif SRRXFLK. Tat signal

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TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description[a]	Source or reference
E. coli strains		
DH5α	*supE44* Δ*lacU169*(ϕ80d*lacZ*ΔM15) *recA1 endA1 hsdR17*(r_K^- m_K^+) *thi-1 gyrA relA1* F^- Δ(*lacZYA-argF*)	Tiangen
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1 lac*(F′ *proAB lacI*^{q}ZΔM15 Tn*10* [Tet^r])	Stratagene
Plasmids		
pUC18	pUC origin of replication, *lac* promoter, Ap^r	TaKaRa
pVLT33	*E. coli-Pseudomonas* shuttle vector, *oriT*, RSF1010, *oriV lacI*^{q}, *tac* promoter, Km^r	10
pSCTorA-GFP	Gene source of TorA signal peptide	22
pMDQ	Source of *mpd* gene	36
pPNCO33	pVLT33 derivative, surface expression vector coding for INPNC-OPH fusion	29
pUTM18	pUC18 derivative, periplasmic expression vector coding for TorA-MPH fusion	This study
pCPO	pVLT33 derivative, control plasmid for expressing a cytosolic OPH	This study
pUM18	pUC18 derivative, control plasmid for expressing a cytosolic MPH	This study
Primers		
P1	GAATTCCTCTAGAGGGTATTAATAATGAACAATAACGATCTCTTT	This study
P2	GGATCCCGCCGCTTGCGCCGCAGTCGC	This study
P3	GGATCCGCCGCACCGCAGGTGCGC	This study
P4	AAGCTTTCACTTGGGGTTGACGAC	This study
P5	GAATTCAGGAAACAATGCAAACGAGAAGG	This study
P6	AAGCTTTCATGACGCCCGCAAGGT	This study
P7	GAATTCCGCCGCACCGCAGGTGCGC	This study

[a] The restriction sites in the primers (5′→3′) are underlined.

peptides consist of three domains: a positively charged N-terminal domain, a hydrophobic domain, and a C-terminal domain (3, 5). In *Escherichia coli*, the membrane-embedded Tat translocase consists of the TatA, TatB, and TatC proteins (19). Once Tat substrates with a twin-arginine motif are specially recognized by the TatBC signal recognition complex, the TatA transport channel complex forms a channel through which substrates are translocated across the cytoplasmic membrane (1, 4, 12).

In this study, *E. coli* was genetically engineered to coexpress OPH and MPH, and it acquired broad-spectrum degradation activity against various OPs. *E. coli* has a complex cell envelope structure that consists of the cytoplasmic membrane, periplasm, cell wall, and outer membrane. To reduce the OP uptake limitation, MPH and OPH were simultaneously targeted to the periplasm and cell surface of *E. coli*, respectively, by fusing MPH to the twin-arginine signal peptide of trimethylamine *N*-oxide reductase (TorA) (22) and OPH to the N- and C-terminal domains of InaV (INPNC) (27). Importantly, we demonstrate here for the first time functional cotranslocation of two distinct OP hydrolases to two independent extracytosolic compartments within a single bacterial cell. The resulting recombinant strain possesses a broader substrate spectrum than strains expressing either one of the hydrolases and, therefore, is a promising candidate for use as a whole-cell biocatalyst for detoxification of OPs.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and culture conditions. All strains, plasmids, and primers used in this study are listed in Table 1. *E. coli* DH5α was used for constructing recombinant plasmids. *E. coli* XL1-Blue was used as a host for recombinant protein expression. XL1-Blue cells carrying pUTM18 and pPNCO33 were grown in Luria-Bertani (LB) medium (25) supplemented with ampicillin (100 μg/ml) and kanamycin (20 μg/ml) at 250 rpm and 37°C. When the culture reached an optical density at 600 nm (OD_{600}) of 0.5, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture broth for induction of recombinant protein expression. After IPTG induction, cells were incubated at 250 rpm and 30°C for 24 h.

Plasmid construction. The *inpnc-opd* fragment was subcloned into EcoRI/HindIII-digested pVLT33 (10) to generate pPNCO33 (29). The nucleic acid corresponding to the entire signal sequence and the first four amino acid residues of mature TorA was amplified by PCR from pSCTorA-GFP (22) using primers P1 and P2. The PCR products were digested with EcoRI and BamHI and ligated into similarly digested pUC18 to generate pUT18. The *mpd* gene was amplified by PCR from pMDQ (36) using primers P3 and P4. The PCR products were digested with BamHI and HindIII and ligated into similarly digested pUT18 to generate pUTM18.

To generate pCPO expressing cytosolic OPH, the *opd* gene was amplified by PCR from pPNCO33 using primers P5 and P6, digested with EcoRI and HindIII, and ligated into similarly digested pVLT33. To generate pUM18 expressing cytosolic MPH, the *mpd* gene was amplified by PCR from pMDQ using primers P7 and P4, digested with EcoRI and HindIII, and ligated into similarly digested pUC18. All plasmid constructs were verified by DNA sequence analysis. Transformation of plasmid into *E. coli* was carried out using the $CaCl_2$ method (25).

Cell fractionation. To demonstrate periplasmic secretion of MPH, cells were fractionated to obtain shocked cell and periplasmic fractions by the cold osmotic shock procedure (9, 33). After disruption of the shocked cells by sonication and a brief clarifying spin, the clarified lysate was ultracentrifuged at 50,000 rpm for 1 h at 4°C, and the supernatant was retained as the cytoplasmic fraction. To verify the surface localization of OPH, cells were fractionated into a soluble fraction and an outer membrane fraction as described previously (20).

Western blot analysis. Subcellular fractionated samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% (wt/vol) acrylamide (25). After electrophoresis, the separated proteins were electroblotted overnight at 40 V onto a nitrocellulose membrane (Millipore, Billerica, MA) with a tank transfer system (Bio-Rad, Hercules, CA) containing a transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol). After nonspecific binding sites were blocked with 3% bovine serum albumin (BSA) in TBST buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Tween 20), the membrane was incubated with either MPH or OPH antisera at a 1:1,000 dilution in TBST buffer. Subsequently, the membrane was incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Promega, Madison, WI) at a 1:2,000 dilution. The membrane was then stained with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) in alkaline phosphatase buffer (100 mM Tris-HCl, 100 mM NaCl; pH 9.0) for visualizing antigen-antibody conjugates.

Immunofluorescence microscopy. Cells were harvested and resuspended (OD_{600}, 0.5) in phosphate-buffered saline (PBS) with 3% BSA. Cells were incu-

bated with OPH antisera at a 1:1,000 dilution for 2 h at 30°C. After the cells were washed with PBS, they were resuspended in PBS with goat anti-rabbit IgG conjugated with rhodamine (Invitrogen, Mukilteo, WA) at a 1:500 dilution and incubated for 1 h at 30°C. Prior to microscopic observation, cells were washed five times with PBS and mounted on poly-L-lysine-coated microscopic slides. Photographs were taken using a fluorescence microscope (Nikon) equipped with fluorescein isothiocyanate and rhodamine filters.

OP hydrolase activity assay. The whole-cell activity of *E. coli* XL1-Blue/pPNCO33/pUTM18 was measured by using five representative OP pesticides as substrates. Hydrolysis of paraoxon, parathion, and methyl parathion was measured spectrophotometrically by monitoring the production of *p*-nitrophenol at 405 nm (ε_{405} = 17,700 M^{-1} cm^{-1}) with a Beckman DU800 spectrophotometer (11). Hydrolysis of fenitrothion was measured by quantifying the formation of 3-methyl-4-nitrophenol at 358 nm (ε_{358} = 18,700 M^{-1} cm^{-1}) (11). Hydrolysis of chlorpyrifos was measured by high-performance liquid chromatography (HPLC) (Agilent 1100) (36). The standard enzyme activity assay was carried out using 100 mM phosphate buffer (pH 7.4) supplemented with 0.2 mM substrate and 100 μl of cells (OD_{600}, 1.0) at 30°C. Activities were expressed in units (1 μmol substrate hydrolyzed per min) per OD_{600} whole cells.

Stability of resting cultures. *E. coli* XL1-Blue/pPNCO33/pUTM18 cells were grown in 50 ml of LB medium supplemented with 0.5 mM IPTG, 100 μg/ml ampicillin, and 20 μg/ml kanamycin for 24 h, washed twice with 50 ml of a 150 mM NaCl solution, resuspended in 5 ml of 100 mM phosphate buffer (pH 7.4), and incubated in a shaker at 30°C. For 2 weeks, 0.1-ml samples were removed each day. Samples were centrifuged and resuspended in 0.1 ml of 100 mM phosphate buffer (pH 7.4). OP hydrolase activity assays were conducted as described above.

Biodegradation of organophosphorus pesticides by recombinant *E. coli* coexpressing OPH and MPH. *E. coli* XL1-Blue/pPNCO33/pUTM18 cells were harvested 24 h after induction and then washed with 100 mM phosphate buffer (pH 7.4) twice and resuspended (OD_{600}, 1.0) in the same buffer. Subsequently, 0.4 mM paraoxon, parathion, methyl parathion, fenitrothion, or chlorpyrifos was added to the cell suspension. Samples were incubated at 30°C for 2 h with shaking, and the residual concentration of pesticide was measured by gas chromatography (36). Other biodegradation experiments were carried out similarly using a mixture of the five pesticides listed above. All pesticides were added at an initial concentration of 0.2 mM. Samples were incubated at 30°C with shaking, and the residual concentration of pesticides was measured at different time points as described above.

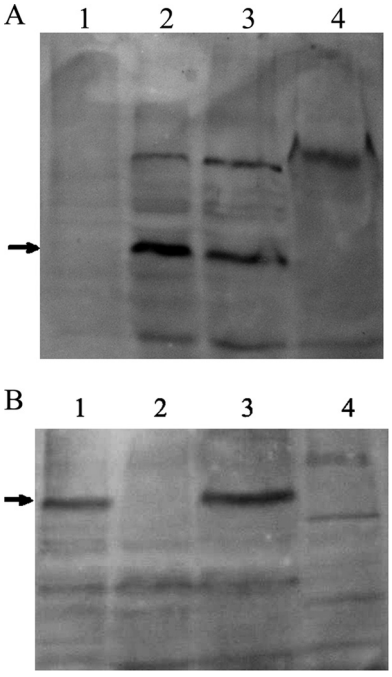

FIG. 1. Western blot analysis of subcellular localization of expressed TorA-MPH and INPNC-OPH in *E. coli* XL1-Blue/pPNCO33/pUTM18. (A) Immunoblot of different subcellular fractions with anti-MPH serum. Lane 1, negative control (XL1-Blue/pUC18); lane 2, total cell lysate; lane 3, periplasmic fraction; lane 4, cytoplasmic fraction. The position of mature-size MPH is indicated by an arrow. (B) Immunoblot of different subcellular fractions with anti-OPH serum. Lane 1, outer membrane fraction; lane 2, soluble fraction; lane 3, total cell lysate; lane 4, negative control (XL1-Blue/pVLT33). The position of INPNC-OPH is indicated by an arrow.

RESULTS AND DISCUSSION

Cotranslocation of MPH to the periplasm and of OPH to the cell surface of *E. coli*. In order to minimize direct competition for the same translocation machinery, MPH and OPH were simultaneously translocated to the periplasm and cell surface of *E. coli*, respectively, by employing the Tat pathway and INP display system. pUTM18, a pUC18-based vector coding for TorA-MPH, was used to target MPH to the periplasmic space of *E. coli*. To generate pUTM18, the *torA-mpd* fusion gene was cloned into the pUC18 vector containing a *lac* promoter, pUC origin of replication, and ampicillin antibiotic resistance marker. For cotranslocation of INPNC-OPH to the cell surface of *E. coli*, we took advantage of pPNCO33 (29), a compatible plasmid that contains a *tac* promoter, RSF origin of replication, and kanamycin antibiotic resistance marker. Since pUTM18 and pPNCO33 contain different replication origins and selection markers, they can coexist in the same cell. Introduction of plasmids was carried out stepwise, and the resulting strain was designated XL1-Blue/pPNCO33/pUTM18.

Expression of TorA-MPH was probed by immunoblotting with MPH antisera, and this mature-size MPH (32 kDa) was detected in total cell lysate (Fig. 1A, lane 2). The TorA signal peptide contains a conserved AxA motif in the C-terminal domain as the recognition site for type I signal peptidases (3, 19, 26). During the release of TorA-MPH into periplasm via membrane-embedded Tat translocase, the precursor was processed to mature-size MPH by cleaving the N-terminal TorA signal peptide. The localization of MPH was determined by immunoblotting subcellular fractionated samples, and the mature-size MPH was found in the periplasmic fraction (Fig. 1A, lane 3). Production of INPNC-OPH was verified by immunoblotting with OPH antisera. A specific band corresponding to INPNC-OPH at 82 kDa was detected in total cell lysate (Fig. 1B, lane 3). Subcellular fractionated samples were probed with OPH antisera, and the 82-kDa band was detected in the outer membrane fraction (Fig. 1B, lane 1). Target proteins were not detected in total cell lysate of the negative controls.

Immunolabeling with specific antibodies or antisera is a useful tool for detecting surface-exposed proteins (29). To confirm the presence of OPH on the *E. coli* surface, immunofluorescence microscopy was used. Cells were probed with OPH antisera and then fluorescently stained with rhodamine-labeled IgG antibody. Since antibodies cannot diffuse through the cell membrane, specific interactions between a target antigen and an antibody should occur only with proteins exposed on the cell surface. When fluorescence microscopy was used, the XL1-Blue/pPNCO33/pUTM18 cells were brightly fluorescent. In contrast, the control XL1-Blue/pCPO cells expressing cytosolic OPH were not immunostained (data not shown). These results indicated that the cell surface of XL1-Blue/pPNCO33/

TABLE 2. OP hydrolase activities of different subcellular fractions of *E. coli* XL1-Blue/pPNCO33/pUTM18 and XL1-Blue/pCPO/pUM18[a]

Strain	OP hydrolase activity (U/OD_{600})				
	Whole cells	Cell lysate	Cytoplasm	Periplasm	Outer membrane
XL1-Blue/pPNCO33/pUTM18	2.38 ± 0.26	3.61 ± 0.32	0.15 ± 0.03	1.26 ± 0.18	1.82 ± 0.21
XL1-Blue/pCPO/pUM18	0.41 ± 0.08	4.21 ± 0.29	3.52 ± 0.24	0.16 ± 0.02	0.12 ± 0.02

[a] Cells were fractionated to obtain different subcellular samples as described in Materials and Methods. The OP hydrolase activities of subcellular fractionated samples were measured using parathion as the substrate at 30°C by monitoring the increases in the linear optical density at 405 nm over time as parathion was hydrolyzed to *p*-nitrophenol (ε_{405} = 17,700 M^{-1} cm^{-1}). Activities were expressed in units (1 μmol *p*-nitrophenol formed per min) per OD_{600} whole cells. The data are means ± standard deviations of three replicates.

pUTM18 was covered with an antibody-rhodamine complex, which confirmed that INPNC-OPH was displayed correctly on the surface of *E. coli* in a stable conformation.

The OP hydrolase activity of subcellular fractionated samples was measured using parathion as the substrate (Table 2). The cell lysate activity of XL1-Blue/pCPO/pUM18 was 10-fold higher than the whole-cell activity, while the cell lysate activity of XL1-Blue/pPNCO33/pUTM18 was 1.5-fold higher than the whole-cell activity. Even though the cell lysate activity of XL1-Blue/pCPO/pUM18 was 1.2-fold higher than that of XL1-Blue/pPNCO33/pUTM18, the latter strain showed 6-fold-higher whole-cell activity than the former strain. In XL1-Blue/pPNCO33/pUTM18 cells, the majority of OP hydrolase activity was detected in the periplasmic fraction and outer membrane fraction (35% and 50% of the cell lysate activity, respectively). In contrast, very little activity was detected in the periplasmic fraction and outer membrane fraction of XL1-Blue/pCPO/pUM18 (4% and 3% of the cell lysate activity, respectively). From all of these results, we concluded that MPH and OPH were simultaneously translocated to the periplasm and cell surface of *E. coli*, respectively, by the Tat pathway and INP display system.

Whole-cell activity. Since the cell membrane acts as a substrate diffusion barrier, the uptake of OP pesticides is a rate-limiting factor in whole-cell biocatalysis systems (24). There have been several reports which have shown the potential of

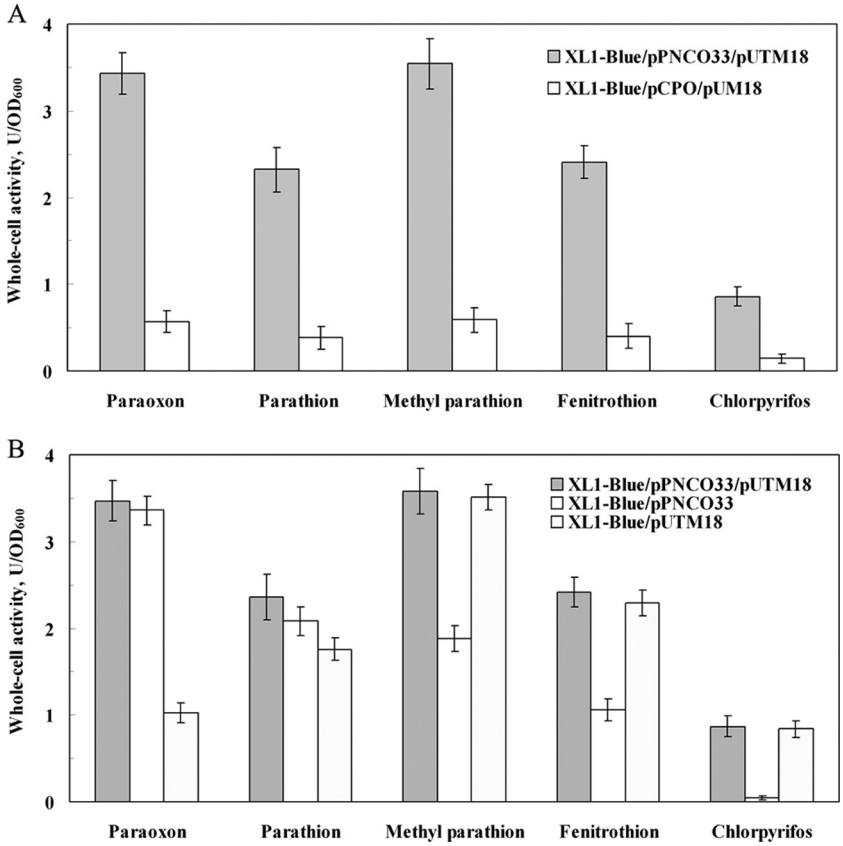

FIG. 2. (A) Whole-cell activities of *E. coli* XL1-Blue/pPNCO33/pUTM18 and XL1-Blue/pCPO/pUM18. (B) Whole-cell activities of XL1-Blue/pPNCO33/pUTM18, XL1-Blue/pPNCO33, and XL1-Blue/pUTM18. OP hydrolase activity was assayed using five representative OP pesticides as substrates as described in Materials and Methods. Activities were expressed in units (1 μmol substrate hydrolyzed per min) per OD_{600} whole cells. The data are means ± standard deviations of three replicates.

transformed microbial species with surface-displayed OPH or periplasmically secreted OPH to accelerate the biodegradation of OP pesticides (15, 16, 24, 29). Five representative OP pesticides were selected for the OP hydrolase activity assay. As shown in Fig. 2A, XL1-Blue/pPNCO33/pUTM18 showed six-fold-higher whole-cell activity with all of the substrates tested than XL1-Blue/pCPO/pUM18 expressing cytosolic OP hydrolases, which indicated that periplasmic secretion and surface display enhanced whole-cell biocatalytic activity by reducing the substrate diffusion barrier. This strategy of combining surface display with periplasmic secretion not only is highly conducive to the biodegradation of OP pesticides but also could be employed for biodegradation of other classes of pollutants.

Although engineered *E. coli* strains expressing OPH or MPH have been developed for detoxifying OPs (8, 16, 24), this is first report of successful construction of recombinant *E. coli* coexpressing OPH and MPH. XL1-Blue/pPNCO33/pUTM18 showed higher activity for dimethyl OPs, such as methyl parathion and fenitrothion, than XL1-Blue/pPNCO33, and it showed higher activity for diethyl OPs, such as paraoxon, than XL1-Blue/pUTM18 (Fig. 2B). XL1-Blue/pPNCO33/pUTM18 showed noticeable activity for chlorpyrifos, while very little activity was detected with XL1-Blue/pPNCO33. These results indicated that the recombinant *E. coli* strain had broad-spectrum activity with various OPs due to coexpression of OPH and MPH.

Directed evolution has recently been used to generate OPH variants with up to 25-fold improvement in the hydrolysis of methyl parathion (6). Cho et al. (7) described directed evolution of OPH to improve the hydrolysis of chlorpyrifos, and up to 700-fold improvement was obtained. The obvious question and challenge are whether similar success could be achieved with other poorly hydrolyzed substrates. Recently, Lan et al. (18) described a novel strategy using a coexpression vector (pETDuet) for construction of an *E. coli* strain coexpressing OPH and carboxylesterase B1. The recombinant *E. coli* strain was capable of producing both enzymes for degradation of organophosphorus, carbamate, and pyrethroid classes of pesticides. This finding suggests that bacteria coexpressing multiple degrading enzymes could be useful in degradation of a variety of pollutants that may occur simultaneously in a contaminated environment.

OPH belongs to the phosphotriesterase family, while MPH belongs to the metallo-β-lactamase superfamily. There are 71 commercial organophosphorus pesticides that are listed by Tomlin (34), 33 and 26 of which contain diethyl and dimethyl alkyl groups, respectively. OPH has been shown to lack any hydrolytic activity with numerous dimethyl OPs (13). MPH has a higher k_{cat}/K_m value for dimethyl OPs than OPH, which is due to the favorable interaction of the substrates with the active sites involved in catalysis (8). Chlorpyrifos accounts for 11% of all of the pesticide used in the United States, and it is a very poorly hydrolyzed substrate for OPH (7, 30). An ability to hydrolyze chlorpyrifos has been demonstrated for the MPH used in this study (36). The use of both hydrolases as biocatalysts is an attractive strategy for detoxification of OPs because of the broader substrate specificity and superior kinetics.

Stability of *E. coli* cultures coexpressing OPH and MPH. Anchorage of foreign proteins on the outer membrane may result in instability of the outer membrane and inhibition of cell growth (24). In this study, a low-copy-number plasmid, pPNCO33, was used to target INPNC-OPH to the *E. coli* surface. Expression of INPNC-OPH is tightly regulated by a *tac* promoter due to the presence of the *lacI*^{q} gene on the plasmid. To test whether display of OPH on the cell surface inhibits cell growth, the growth kinetics of XL1-Blue/pPNCO33/pUTM18 and XL1-Blue/pVLT33/pUC18 were compared. No growth inhibition was observed for cells with surface-displayed OPH. XL1-Blue/pPNCO33/pUTM18 showed the same growth profile as XL1-Blue/pVLT33/pUC18 (Fig. 3A). The two cultures reached the same final cell density after 48 h of incubation. To monitor the stability of suspended cultures, whole-cell activity was determined periodically using parathion as the substrate over a 2-week period. As shown in Fig. 3B, the whole-cell activity of XL1-Blue/pPNCO33/pUTM18 remained at essentially the original level over the 2-week period. These results showed that surface display of OPH did not disturb the membrane structure or cause host growth defects, which suggested that the metabolic burden placed on the cell could be alleviated by selection of compatible surface anchors and/or optimization of expression regulation systems. The stability of the cells observed here is in line with the results of previous studies in which the INP system was used for surface display of other proteins (14, 20). All of these results collectively suggest that

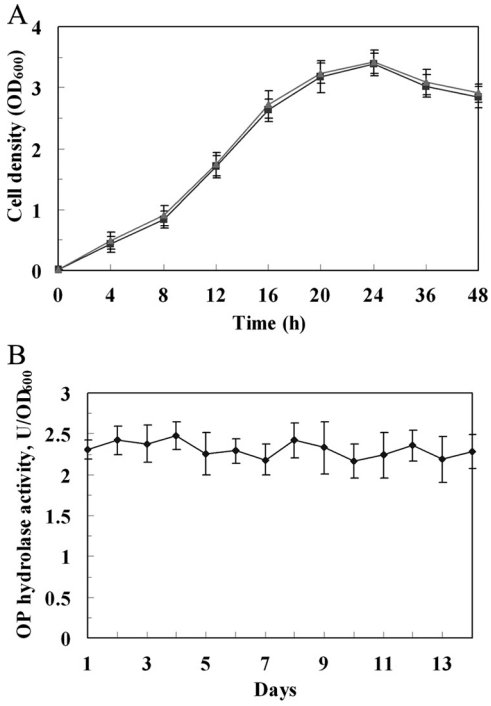

FIG. 3. (A) Time courses for the growth of *E. coli* XL1-Blue/pPNCO33/pUTM18 (■) and XL1-Blue/pVLT33/pUC18 (▲). *E. coli* was incubated in Luria-Bertani (LB) medium supplemented with either ampicillin (100 μg/ml) or kanamycin (20 μg/ml) at 37°C for 4 h and then induced with 0.5 mM IPTG and incubated at 30°C for 44 h. The cell concentration was determined by measuring the optical density at 600 nm (OD_{600}) of the culture broth. (B) Whole-cell activity in suspended *E. coli* XL1-Blue cultures coexpressing OPH and MPH. OP hydrolase activity was measured with parathion as the substrate. The data are means ± standard deviations of three replicates.

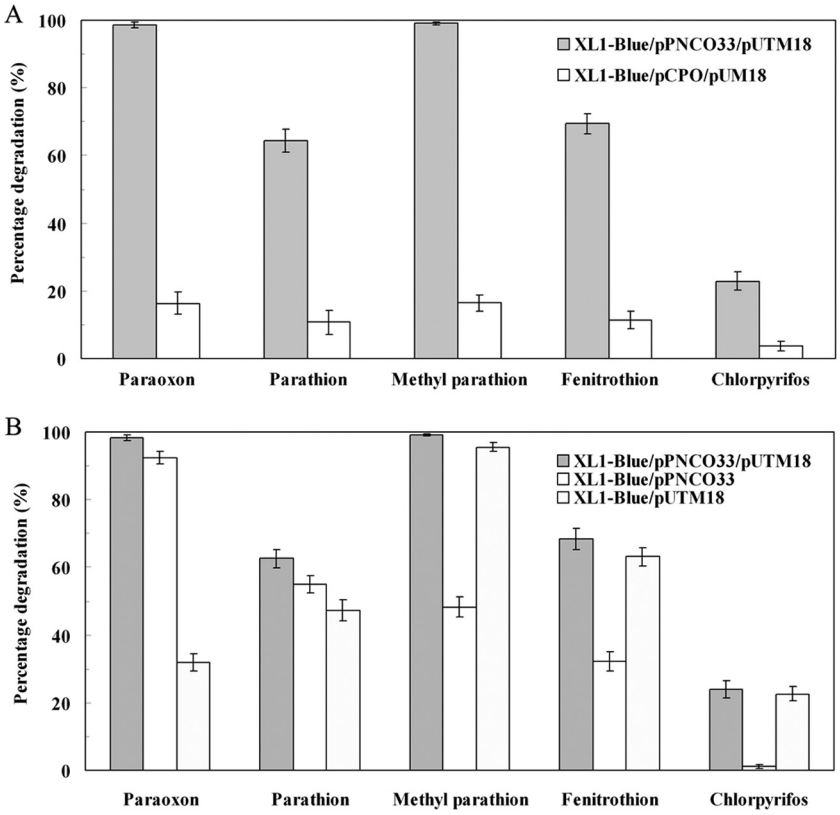

FIG. 4. (A) Biodegradation of OP pesticides by *E. coli* XL1-Blue/pPNCO33/pUTM18 and XL1-Blue/pCPO/pUM18. (B) Biodegradation of OP pesticides by XL1-Blue/pPNCO33/pUTM18, XL1-Blue/pPNCO33, and XL1-Blue/pUTM18. All pesticides were added at an initial concentration of 0.4 mM. The residual concentration of pesticide was measured by gas chromatography as described in Materials and Methods. The data are means ± standard deviations of three replicates.

the recombinant *E. coli* strain could be employed as a whole-cell biocatalyst for detoxification of OPs. The resulting live biocatalysts are also considerably more stable and robust than purified OPH or MPH in terms of the long-term stability and simplification of the protein purification step.

Biodegradation of organophosphorus pesticides by recombinant *E. coli* coexpressing OPH and MPH. To investigate the ability of the recombinant *E. coli* strain to degrade various OP pesticides, the rates of degradation were measured for five representative OP pesticides. XL1-Blue/pPNCO33/pUTM18 degraded the pesticides sixfold faster than XL1-Blue/pCPO/pUM18 expressing cytosolic OP hydrolases (Fig. 4A), which indicated that this barrier of substrate transport could be eliminated by periplasmic secretion and surface display. XL1-Blue/pPNCO33/pUTM18 degraded dimethyl OPs, such as methyl parathion and fenitrothion, faster than XL1-Blue/pPNCO33, and it degraded diethyl OPs, such as paraoxon, faster than XL1-Blue/pUTM18 (Fig. 4B). Very little degradation of chlorpyrifos was observed with XL1-Blue/pPNCO33. The change in degradation rate is consistent with the complementary substrate specificities of OPH and MPH. XL1-Blue/pPNCO33/pUTM18 could efficiently degrade a mixture of the five pesticides mentioned above because it had an enlarged substrate spectrum. All pesticides (0.2 mM each) could be degraded completely by the recombinant *E. coli* strain within 5 h. In contrast, the pesticide mixture could not be degraded effectively by XL1-Blue/pPNCO33 or XL1-Blue/pUTM18. These results highlight the potential of the coexpression strain for use in the biodegradation of a pesticide mixture containing diethyl and dimethyl OPs. The coexpression strain loses its degradation ability because of the loss of plasmids after successive plating on nonselective media without antibiotics. This limitation needs to be addressed in future studies.

The use of engineered microorganisms as bioremediating biocatalysts is a promising approach for removal of organic pollutants in the environment (30, 32). *E. coli* is still the most commonly used host for recombinant protein expression due to its ability to grow rapidly and densely on numerous inexpensive substrates, its well-characterized genetics and cellular properties, and the availability of increasingly numerous cloning vectors and mutant strains (2). In field studies, recombinant *E. coli* strains overexpressing atrazine chlorohydrolase have been used for *in situ* bioremediation of atrazine-contaminated soil (32). Recently, engineered *E. coli* strains expressing OPH or MPH have been tested for the ability to degrade different organophosphorus pesticides (16, 24, 36). In 2008, a bioreactor inoculated with MPH-expressing *E. coli* was developed and used successfully for removal of methyl parathion from artificial off-gas (21). We are currently investigating the degradation of a wide variety of pesticides in a bioreactor with recombinant *E. coli* cells coexpressing OPH and MPH.

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