DOI:10.1021/jf101105s

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Genetic Engineering of *Stenotrophomonas* Strain YC-1 To Possess a Broader Substrate Range for Organophosphates

Chao Yang,[†,‡] Cunjiang Song,[‡] Ashok Mulchandani,*[,§] and Chuanling Qiao*[,†]

[†]State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China, [‡]Department of Microbiology, College of Life Sciences, Nankai University, Tianjin 300071, China, and [§]Department of Chemical and Environmental Engineering, University of California, Riverside, California 92521

In this work, *Stenotrophomonas* sp. strain YC-1, a native soil bacterium that produces methyl parathion hydrolase (MPH), was genetically engineered to possess a broader substrate range for organophosphates (OPs). A surface anchor system derived from the truncated ice nucleation protein (INPNC) from *Pseudomonas syringae* was used to target organophosphorus hydrolase (OPH) onto the cell surface of strain YC-1, reducing the potential substrate uptake limitation. The surface localization of INPNC−OPH was verified by cell fractionation, Western blot, proteinase accessibility, and immunofluorescence microscopy. No growth inhibition was observed for the engineered strain, and suspended cultures retained almost 100% activity over a period of 2 weeks. Concomitant expression of OPH in strain YC-1 resulted in a recombinant strain capable of simultaneously degrading diethyl and dimethyl OPs. A mixture of six OP pesticides (0.2 mM each) could be degraded completely within 5 h. The broader substrate specificity in combination with the rapid degradation rate makes this engineered strain a promising candidate for *in situ* remediation of OP-contaminated sites.

KEYWORDS: Organophosphate detoxification; organophosphorus hydrolase; methyl parathion hydrolase; *Stenotrophomonas*

INTRODUCTION

Synthetic organophosphates (OPs) are widely used to control agricultural pests and account for ∼38% of the total pesticides used globally (*1*). In the United States alone, over 40 million kilograms of OP pesticides are consumed annually (*2*). OPs are acute neurotoxins because they inhibit acetylcholinesterase in the central nervous system synapses, leading to a subsequent loss of nerve function and eventual death (*3*). OP pesticides are mostly liposoluble and pose a hazard to humans through accumulation in the food chain. For some pesticides, up to 90% of the application amount may volatize from agricultural fields into the air, which constitutes a large source of potential human exposure.

Organophosphorus hydrolase (OPH), isolated from natural soil microorganisms *Pseudomonas diminuta* MG and *Flavobacterium* sp. strain ATCC 27551, has been shown to hydrolyze different OP pesticides as well as the more toxic chemical-warfare agents such as Sarin, Soman, and VX (*4*, *5*). Hydrolysis of OPs by OPH dramatically reduces their toxicity (*3*). Unfortunately, the rates of hydrolysis by OPH differ dramatically for individual members of the family of OPs. For example, methyl parathion and chlorpyrifos are hydrolyzed by OPH 30- and 1200-fold slower than is the preferred substrate, paraoxon (*6*, *7*). In 2001, an OP degradation gene (*mpd*) encoding methyl parathion hydrolase (MPH) was isolated from methyl parathion-degrading *Plesiomonas* sp. strain M6, but it lacks sequence homology with the *opd* gene that encodes OPH (*8*).

Most microorganisms that produce OPH are Gram-negative bacteria, and their OPH is located within the cells (*1*). The ability of the cell membrane to act as a permeability barrier limits interaction between the pesticides and OPH residing within the cell, which dramatically reduces whole-cell catalytic efficiency. However, whole-cell activity can be improved by targeting OPH to the periplasm or cell surface (*2*, *9*−*11*).

Ice nucleation protein (INP) is an outer membrane protein from *Pseudomonas syringae*, which accelerates ice crystal formation in supercooled water. INP has a multidomain organization with an N-terminal domain containing three or four transmembrane spans, a C-terminal domain, and a highly repetitive central domain for ice nucleation (*12*, *13*). INP has been used to display heterologous proteins, such as levansucrase (*14*), carboxymethylcellulase (*15*), salmobin (*16*), HIV-1 gp120 (*17*), green fluorescent protein (*18*), chitinase (*19*), and cytochrome P450 oxidoreductase (*20*), on the cell surface of *Escherichia coli*. INP-mediated cell surface display can be achieved using either full-length INP sequences or truncated INP sequences (*14*−*20*).

A recombinant *E. coli* strain was genetically engineered to coproduce OPH and carboxylesterase B1 for the simultaneous degradation of organophosphorus, carbamate, and pyrethroid classes of pesticides (*21*). However, *E. coli* 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.

*To whom correspondence should be addressed: A.M. (phone, 951-827-6419; fax, 951-827-5696; e-mail, adani@engr.ucr.edu); C.Q. (phone, 86-10-64807191; fax, 86-10-64807099; e-mail, qiaocl@ioz.ac.cn).

 Published on Web 05/10/2010

We previously isolated chlorpyrifos-degrading *Stenotrophomonas* sp. strain YC-1, and this strain contains a chromosome-based *mpd* gene that encodes MPH (*22*). In this work, we demonstrate that OPH could be functionally displayed on the cell surface of strain YC-1 using the INPNC anchor. Since OPH and MPH have different substrate specificities, the engineered strain was endowed with the capability to rapidly degrade diethyl and dimethyl OPs.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions. *Stenotrophomonas* sp. strain YC-1 isolated from activated sludge by selective enrichment with chlorpyrifos (*22*) was used as host strain for cell surface display of OPH. A surface expression vector, pPNCO33 (*2*), coding for INPNC−OPH was used to target OPH onto the cell surface. The *inpnc−opd* fragment was PCR amplified from pINCOP (*2*) and subcloned into *Eco*RI/*Hin*dIII-digested pVLT33 (*23*), an *E. coli−Pseudomonas* shuttle vector, to generate pPNCO33. Plasmid pCPO (*24*) was used for the production of cytosolic OPH.

Transformation of plasmid into strain YC-1 was carried out using the $CaCl_2-MgCl_2$ method (*25*). Strains bearing plasmids were grown at 30 °C in Luria−Bertani (LB) medium (*26*) supplemented with kanamycin to a final concentration of 50 μg/mL. Expression of INPNC−OPH and MPH was induced by adding 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and 10 μg/mL methyl parathion to the culture grown to an optical density at 600 nm (OD_{600}) of 0.4. After induction, cells were grown for 24 h at 30 °C.

Cell Fractionation. To verify the surface localization of OPH, cells were fractionated to yield soluble fraction and outer membrane fraction (*18*, *25*). After disruption of the cells by sonication and a brief clarifying spin, the clarified lysate was ultracentrifuged at 50000 rpm for 1 h at 4 °C, and the supernatant was retained as the soluble fraction. The pellet (total membrane fraction) was resuspended with PBS containing 0.01 mM $MgCl_2$ and 2% Triton X-100 for solubilizing the inner membrane and was incubated for 30 min at room temperature, and then the outer membrane fraction was repelleted by ultracentrifugation. To demonstrate periplasmic secretion of MPH, cells were fractionated to yield cytoplasmic and periplasmic fractions by the cold osmotic shock procedure (*27*).

SDS−PAGE and Western Blot Analysis. Subcellular fractionated samples were mixed with sample buffer (200 mM Tris-HCl, pH 6.8, 8% SDS, 0.04% bromophenol blue, 8% β-mercaptoethanol, 40% glycerol), boiled for 5 min, and analyzed by 12% sodium dodecyl sulfate−polyacyrlamide gel electrophoresis (SDS−PAGE) (*26*). Proteins were electroblotted onto nitrocellulose membranes using a tank transfer system (Bio-Rad) at 40 V for 3 h. Blotted membranes were blocked in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) with 3% bovine serum albumin (BSA). For immunodetection, membranes were incubated with rabbit anti-OPH serum diluted (1:1000) in TBST with 3% BSA for 3 h. After being washed with TBST, secondary antibody was added and incubated for 2 h at room temperature. Antigen−antibody conjugates were visualized by reaction with alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody diluted (1:2000) in TBST. After being washed with PBS, a color reaction was achieved by using the Immun-Blot BCIP/NBT kit (Bio-Rad). Subcellular localization of MPH was determined by Western blot with rabbit anti-MPH serum (1:500 dilution) and alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (1:1000 dilution) as described previously (*27*).

Immunofluorescence Microscopy. Cells carrying pPNCO33 were harvested and resuspended (OD_{600} = 0.5) in PBS with 3% BSA. Cells were then incubated with rabbit anti-OPH serum diluted (1:500) in PBS for 3 h at 30 °C. After being washed with PBS, the cells were resuspended in PBS with goat anti-rabbit IgG conjugated with rhodamine (1:100 dilution) and incubated for 2 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 FITC and rhodamine filters.

OP Hydrolase Assay. Whole-cell OP hydrolase activity was measured using paraoxon, parathion, methyl parathion, fenitrothion, chlorpyrifos, or diazinon as the substrate. Hydrolysis of paraoxon, parathion, and methyl parathion was measured spectrophotometrically by monitoring the production of *p*-nitrophenol at 405 nm (ε_{405} = 17700 M^{-1} cm^{-1}) with a Beckman DU800 spectrophotometer (*28*). Hydrolysis of fenitrothion was measured by quantifying the formation of 3-methyl-4-nitrophenol at 358 nm (ε_{358} = 18700 M^{-1} cm^{-1}) (*28*). Hydrolysis of chlorpyrifos and diazinon was measured by gas chromatography as described below. The standard enzyme activity assay was carried out using 50 mM citrate−phosphate buffer (pH 8.0) supplemented with 50 μM $CoCl_2$, 0.2 mM substrate, and 100 μL of cells (OD_{600} = 1.0) at 30 °C. Activities are expressed as units (1 μmol of substrate hydrolyzed per minute) per OD_{600} whole cells.

Proteinase Accessibility Assay. Cells carrying pPNCO33 were centrifuged and resuspended (OD_{600} = 1.0) in 1 mL of 15% sucrose, 15 mM Tris-HCl, and 0.1 mM EDTA, pH 7.8. Samples were incubated for 3 h with 5 μL of 20 mg/mL proteinase K at room temperature. Proteinase K treated cells were assayed for Western blot and immunofluorescence microscopy as described above.

Stability Study of Resting Cultures. Cells carrying pPNCO33 were grown in 50 mL of LB medium supplemented with 1 mM IPTG, 10 μg/mL methyl parathion, and 50 μg/mL kanamycin for 24 h, washed twice with 50 mL of 150 mM NaCl solution, resuspended in 5 mL of 50 mM citrate−phosphate buffer (pH 8.0) with 50 μM $CoCl_2$, and incubated in a shaker at 30 °C. Over a 2 week duration, 0.1 mL of samples was removed each day. Samples were centrifuged and resuspended in 0.1 mL of 50 mM citrate−phosphate buffer (pH 8.0) with 50 μM $CoCl_2$. OP hydrolase activity assays were conducted as described above.

OP Pesticide Degradation. *Stenotrophomonas* sp. strain YC-1 carrying pPNCO33 was inoculated at OD_{600} = 0.01 into LB medium (*26*) supplemented with 1 mM IPTG, 10 μg/mL methyl parathion, and 50 μg/mL kanamycin and incubated for 24 h at 30 °C and 300 rpm. The cells were harvested and then washed with 50 mM citrate−phosphate buffer (pH 8.0) twice and resuspended in the same buffer with 50 μM $CoCl_2$. For the OP degradation test, paraoxon, parathion, methyl parathion, fenitrothion, diazinon, and chlorpyrifos (0.2 mM each) were added to cell suspension (OD_{600} = 1.0). Samples were incubated at 30 °C and 150 rpm and taken at different time points. Remaining pesticide in the suspension was extracted with trichloromethane, and the extracts were then dried over anhydrous Na_2SO_4. Samples of 1 μL (diluted if necessary) were analyzed using a Hewlett-Packard 5890 II GC equipped with NPD detector and a capillary HP-1 column. The concentration of pesticide was determined by comparing the peak area of the samples to a standard curve.

RESULTS AND DISCUSSION

Surface Localization of INPNC−OPH in *Stenotrophomonas* sp. Strain YC-1. Both InaK from *P. syringae* KCTC1832 and InaV from *P. syringae* INA5 have been used for the display of heterologous proteins on the cell surface (*14*, *29*). Even though functionally similar, there is only 77% sequence homology between the two proteins. Most of the differences occur at the critical N-terminal domain, which interacts with the phospholipid moiety of the outer membrane. Compared to InaK anchor, the use of InaV anchor resulted in 100-fold higher OPH activity in *Moraxella* sp. (*2*). In the present study, to investigate the feasibility of targeting OPH onto the cell surface of strain YC-1, the truncated InaV protein (INPNC) (*29*) was used as a surface-anchoring motif. For expression of INPNC−OPH, the *inpnc−opd* fragment was subcloned into pVLT33 (*23*), a medium-copy-number vector, to give pPNCO33. The broad-host-range vector, pVLT33, is an RSF1010 derivative and therefore is able to replicate in a wide variety of Gram-negative bacteria (*23*). Expression of INPNC−OPH was tightly regulated by a *tac* promoter due to the presence of the $lacI^q$ gene on the plasmid.

Expression of INPNC−OPH in strain YC-1 was verified by Western blot with anti-OPH serum. A band corresponding to INPNC−OPH at 82 kDa was detected in whole-cell lysates (**Figure 1**, lane 3). The localization of INPNC−OPH in the outer membrane was demonstrated by immunoblotting of subcellular fractionated samples with anti-OPH serum (**Figure 1**, lane 2). Over 90% of OPH activity was detected in the outer membrane

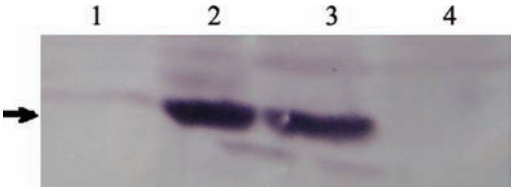

Figure 1. Western blot analysis for subcellular localization of INPNC−OPH in *Stenotrophomonas* sp. strain YC-1 carrying pPNCO33. Lane 1, soluble fraction; lane 2, outer membrane fraction; lane 3, whole-cell lysates; lane 4, proteinase K digested outer membrane proteins. OPH antiserum was used at a 1:1000 dilution.

fraction of cells expressing INPNC−OPH. In parallel, more than 90% of OPH activity was present on the cell surface as judged from the ratio of whole-cell activity to cell lysate activity.

Proteinases cannot penetrate the outer membrane, and therefore, only surface-exposed proteins can be degraded by proteinases (*9*, *18*). A proteinase accessibility assay can be used to provide evidence for the surface localization of OPH. After the treatment of cells with proteinase K, the fractionated outer membrane samples were probed with anti-OPH serum. As expected, no target proteins were detected in the outer membrane fraction (**Figure 1**, lane 4) because of the degradation of surface-exposed OPH by proteinase K.

Immunolabeling with specific antibodies or antisera is a useful tool to detect surface-exposed proteins (*2*, *25*). To confirm the presence of INPNC−OPH on the cell surface, cells were probed with rabbit anti-OPH serum as a primary antibody and then fluorescently stained with rhodamine-labeled goat anti-rabbit IgG antibody. Since antibodies cannot diffuse through the outer membrane, specific interactions should only occur with proteins exposed on the cell surface. Under a fluorescence microscope, cells carrying pPNCO33 were brightly fluorescent (**Figure 2B**), while control cells expressing OPH intracellularly (pCPO) were not immunostained at all (**Figure 2A**).

Cells carrying pPNCO33 were incubated for 3 h with proteinase K and then immunolabeled with anti-OPH serum and rhodamine-conjugated IgG secondary antibody. As a result, proteinase K treated cells were not immunostained completely, indicating that surface-exposed OPH had been removed by the treatment with proteinase K. From all of these results, we concluded that OPH was displayed functionally on the cell surface using the INPNC anchor.

OP Hydrolase Activity. *Stenotrophomonas* sp. strain YC-1 with surface-expressed OPH showed 6-fold higher whole-cell OPH activity with all of the pesticides mentioned above than the same strain expressing cytosolic OPH (data not shown). This improvement in whole-cell activity is attributed to enhanced interaction between the pesticides and surface-exposed OPH. Periplasmic secretion of OPH or MPH can be an alternative strategy for alleviating the substrate uptake limitation (*10*, *11*, *27*). MPH can be produced at high levels by wild-type strain YC-1 and exported to the periplasmic space of this strain (*22*). As expected, MPH was also found in the periplasmic fraction of the engineered strain YC-1 as demonstrated by Western blot with anti-MPH serum (**Figure 3**). The engineered strain capable of targeting OPH and MPH to the extracytosolic compartment overcomes the mass transport limitation of OP pesticides across the cell membrane and, therefore, can be used as a whole-cell biocatalyst for detoxification of OPs.

The engineered strain YC-1 with surface-expressed OPH showed higher hydrolytic activity for diethyl OPs, such as paraoxon, parathion, and diazinon, than wild-type strain YC-1 (**Figure 4**). Compared to engineered microbes expressing OPH

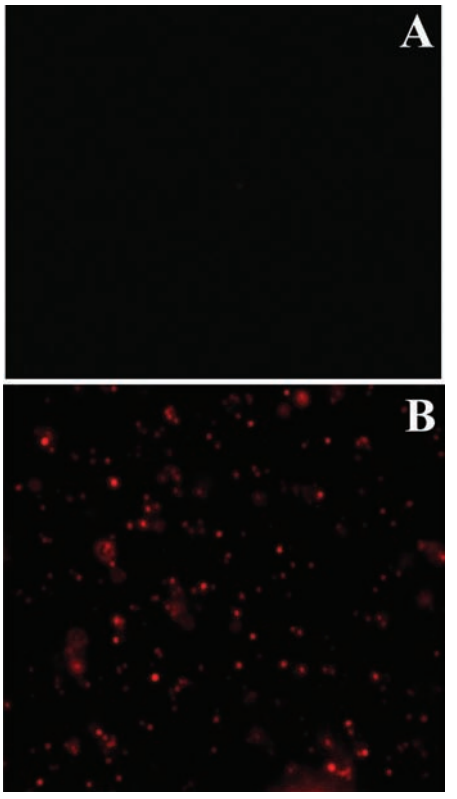

Figure 2. Immunofluorescence micrographs of *Stenotrophomonas* sp. strain YC-1 carrying pCPO (**A**) or pPNCO33 (**B**). Cells were probed with rabbit anti-OPH serum and fluorescently stained with goat anti-rabbit IgG-rhodamine conjugate.

Figure 3. Western blot analysis for subcellular localization of MPH in *Stenotrophomonas* sp. strain YC-1 carrying pPNCO33. Cells were fractionated to yield cytoplasmic and periplasmic samples (C and P) and immunoblotted using anti-MPH serum (1:500 dilution).

alone (*2*, *9*, *25*), the engineered strain YC-1 with native MPH showed higher hydrolytic activity for dimethyl OPs, such as methyl parathion and fenitrothion. Chlorpyrifos is a very poorly hydrolyzed substrate for OPH (*7*), while MPH was shown to effectively hydrolyze chlorpyrifos (*22*, *30*). The engineered strain YC-1 with native MPH showed noticeable hydrolytic activity for chlorpyrifos (**Figure 4**). The engineered strain capable of coproducing OPH and MPH has a broader substrate range than strains expressing either one of the hydrolases. Here we demonstrate for the first time the simultaneous production of two OP-hydrolyzing enzymes in an engineered soil bacterium.

There are 71 commercial organophosphorus pesticides that are listed by Tomlin (*31*), 33 and 26 of which contain diethyl and dimethyl ester groups, respectively. OPH has been shown to lack any hydrolytic activity with numerous dimethyl OPs (*32*). Directed evolution has recently been used to generate OPH variants with up to 25-fold improvement in the hydrolysis of methyl parathion (*6*). The obvious question and challenge are whether similar success could be achieved with other poorly hydrolyzed substrates. MPH differs in the substrate specificity from OPH. For example, MPH is capable of hydrolyzing dimethyl OPs at a higher rate than OPH (*8*). The coproduction of OPH and MPH in

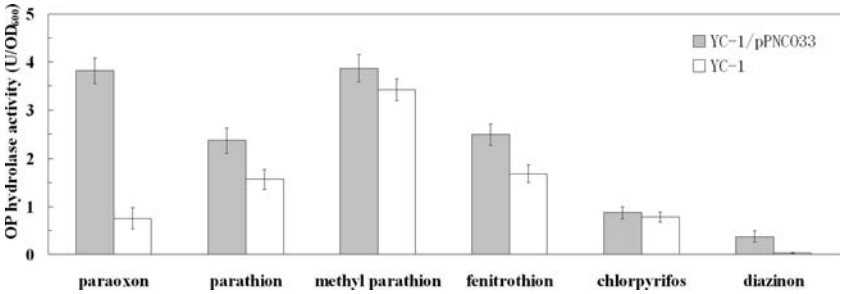

Figure 4. Whole-cell activity of *Stenotrophomonas* sp. strain YC-1 carrying pPNCO33 and of wild-type strain YC-1. Expression of both OPH and MPH was induced with 1 mM IPTG and 10 μg/mL methyl parathion, and OP hydrolase activity was measured by using paraoxon, parathion, methyl parathion, fenitrothion, chlorpyrifos, or diazinon as the substrate. Activities are expressed as units (1 μmol of substrate hydrolyzed per minute) per OD_{600} whole cells. The data are means ± standard deviations of three replicates.

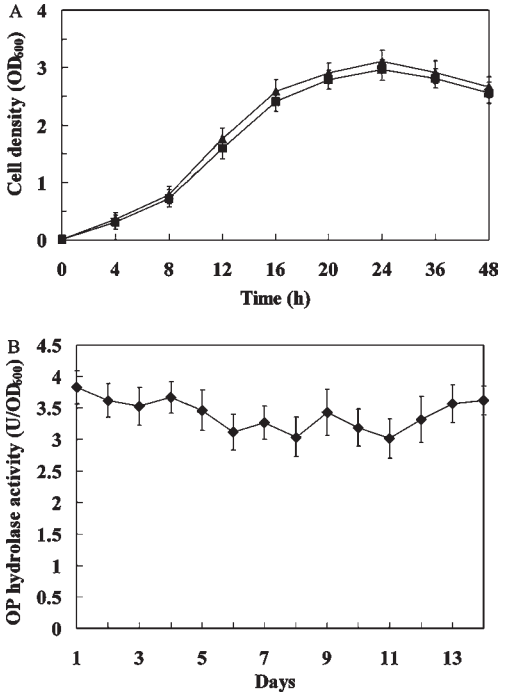

Figure 5. (**A**) Time courses for the growth of *Stenotrophomonas* sp. strain YC-1 carrying pPNCO33 (■) or pVLT33 (▲). Cells were incubated in Luria−Bertani (LB) medium supplemented with 50 μg/mL kanamycin at 30 °C for 4 h and then induced with 1 mM IPTG and incubated at 30 °C for 44 h. The cell concentration was determined by measuring the optical density at 600 nm (OD_{600}) of the culture broth. (**B**) Whole-cell activity in suspended cultures expressing INPNC−OPH. OP hydrolase activity was measured using paraoxon as the substrate. The data are means ± standard deviations of three replicates.

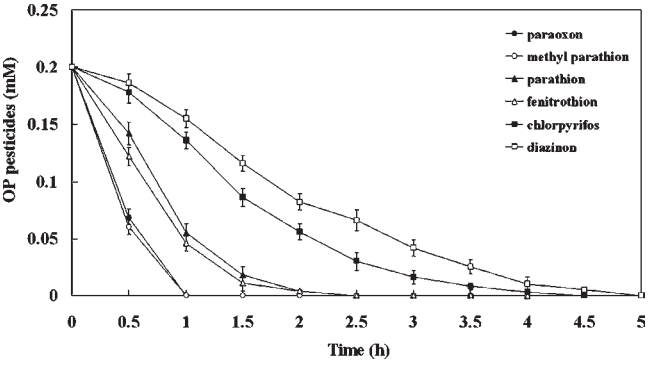

Figure 6. Degradation of a mixture of OP pesticides by *Stenotrophomonas* sp. strain YC-1 carrying pPNCO33. Cell suspensions were incubated with a mixture of six OP pesticides (0.2 mM each) at 30 °C and 150 rpm. Samples were taken at different time points, and the residual concentration of pesticide was measured by gas chromatography. The data are means ± standard deviations of three replicates.

a single microbe may provide an alternative to protein engineering for the acquisition of a broader substrate range. OPAA isolated from *Alteromonas* sp. has higher catalytic activity against G-agents (*33*), which may develop efficient enzymatic destruction methods for chemical warfare agents.

Stability Study of Resting Cultures. Anchorage of heterologous proteins on the outer membrane may result in instability of the outer membrane and inhibition of cell growth (*34*). To test whether surface display of OPH inhibits cell growth, the growth kinetics of cells carrying pPNCO33 or pVLT33 were compared. No growth inhibition was observed for cells expressing INPNC−OPH. The two cultures reached the same final cell density after 48 h of incubation (**Figure 5A**). To monitor the stability of suspended cultures, whole-cell activity was determined periodically over a 2 week period. As shown in **Figure 5B**, the OP hydrolase activity of whole cells remained at essentially the original level over the 2 week period. These results show that surface display of OPH did not disturb the membrane structure or cause host growth defects.

Degradation of OP Pesticides by Engineered *Stenotrophomonas* sp. Strain YC-1. A mixture of paraoxon, parathion, methyl parathion, fenitrothion, diazinon, and chlorpyrifos was used to assess the degradation capability of the engineered strain YC-1. As shown in **Figure 6**, all pesticides (0.2 mM each) could be degraded completely by the engineered strain within 5 h, which indicates that the engineered strain acquires an enlarged substrate range and can be employed for the degradation of a mixture containing diethyl and dimethyl OPs.

Bioremediation exploits the catabolic diversity of microorganisms to transform contaminants into less harmful compounds. Polluted environment often lacks necessary nutrients, which prevents the proliferation of engineered microbes released in the environment. Byproducts released from hydrolysis of OPs may inhibit the growth of microbes because of their toxicity to cells (*2*, *22*). *Stenotrophomonas* sp. strain YC-1, isolated from the sludge of the wastewater treating system of an OP pesticide manufacturer, has been successfully used for the remediation of chlorpyrifos-contaminated soil (*22*). Members of the genus *Stenotrophomonas* were shown to degrade a wide range of recalcitrant pollutants, and these bacteria are robust and ubiquitous in soil (*35*, *36*). Strain YC-1 can utilize OPs as its sole source of carbon for growth and has an ability to tolerate high concentrations of toxic hydrolytic products (*22*). In particular, the

engineered strain YC-1 capable of coproducing OPH and MPH has enormous potential for the degradation of a mixture of OP pesticides. These desirable traits make this bacterium very attractive as a platform for *in situ* bioremediation of OP-contaminated soil. This feasibility is currently under investigation.

LITERATURE CITED

(1) Singh, B. K.; Walker, A. Microbial degradation of organophosphorus compounds. *FEMS Microbiol. Rev*. **2006**, *30*, 428–471.

(2) Shimazu, M.; Mulchandani, A.; Chen, W. Simultaneous degradation of organophosphorus pesticides and *p*-nitrophenol by a genetically engineered *Moraxella* sp. with surface-expressed organophosphorus hydrolase. *Biotechnol. Bioeng*. **2001**, *76*, 318–324.

(3) Sogorb, M. A.; Vilanova, E.; Carrera, V. Future application of phosphotriesterases in the prophylaxis and treatment of organophosphorus insecticide and nerve agent poisoning. *Toxicol. Lett*. **2004**, *151*, 219–233.

(4) Serdar, C. M.; Gibson, D. T. Enzymatic hydrolysis of organophosphates: cloning and expression of a parathion hydrolase gene from *Pseudomonas diminuta*. *Bio/Technology* **1985**, *3*, 567–571.

(5) Mulbry, W. W.; Hams, J. F.; Kearney, P. C.; Nelson, J. O.; McDaniel, C. S.; Wild, J. R. Identification of a plasmid-borne parathion hydrolase gene from *Flavobacterium* sp. by Southern hybridization with *opd* from *Pseudomonas diminuta*. *Appl. Environ. Microbiol*. **1986**, *51*, 926–930.

(6) Cho, C. M.-H.; Mulchandani, A.; Chen, W. Bacterial cell surface display of organophosphorus hydrolase for selective screening of improved hydrolysis of organophosphate nerve agents. *Appl. Environ. Microbiol*. **2002**, *68*, 2026–2030.

(7) Cho, C. M.-H.; Mulchandani, A.; Chen, W. Altering the substrate specificity of organophosphorus hydrolase for enhanced hydrolysis of chlorpyrifos. *Appl. Environ. Microbiol*. **2004**, *70*, 4681–4685.

(8) Cui, Z. L.; Li, S. P.; Fu, G. P. Isolation of methyl parathion-degrading strain M6 and cloning of the methyl parathion hydrolase gene. *Appl. Environ. Microbiol*. **2001**, *67*, 4922–4925.

(9) Richins, R. D.; Kaneva, I.; Mulchandani, A.; Chen, W. Biodegradation of organophosphorus pesticides by surface-expressed organophosphorus hydrolase. *Nat. Biotechnol*. **1997**, *15*, 984–987.

(10) Kang, D. G.; Lim, G.-B.; Cha, H. J. Functional periplasmic secretion of organophosphorous hydrolase using the twin-arginine translocation pathway in *Escherichia coli*. *J. Biotechnol*. **2005**, *118*, 379–385.

(11) Kang, D. G.; Choi, S. S.; Cha, H. J. Enhanced biodegradation of toxic organophosphate compounds using recombinant *Escherichia coli* with Sec pathway-driven periplasmic secretion of organophosphorus hydrolase. *Biotechnol. Prog*. **2006**, *22*, 406–410.

(12) Kozloff, L. M.; Turner, M. A.; Arellano, F. Formation of bacterial membrane ice-nucleation lipoglycoprotein complexes. *J. Bacteriol*. **1991**, *173*, 6528–6536.

(13) Wolber, P. K. Bacterial ice nucleation. *Adv. Microb. Physiol*. **1993**, *34*, 203–237.

(14) Jung, H. C.; Lebeault, J. M.; Pan, J. G. Surface display of *Zymomonas mobilis* levansucrase by using the ice-nucleation protein of *Pseudomonas syringae*. *Nat. Biotechnol*. **1998**, *16*, 576–580.

(15) Jung, H. C.; Park, J. H.; Park, S. H.; Lebeault, J. M.; Pan, J. G. Expression of carboxymethylcellulase on the surface of *Escherichia coli* using *Pseudomonas syringae* ice nucleation protein. *Enzyme Microb. Technol*. **1998**, *22*, 348–354.

(16) Jeong, H. S.; Yoo, S. K.; Kim, E. J. Cell surface display of salmobin, a thrombin-like enzyme from *Agkistrodon halys* venom on *Escherichia coli* using ice nucleation protein. *Enzyme Microb. Technol*. **2001**, *28*, 155–160.

(17) Kwak, Y. D.; Yoo, S. K.; Kim, E. J. Cell surface display of human immunodeficiency virus type 1 gp120 on *Escherichia coli* by using ice nucleation protein. *Clin. Diagn. Lab. Immunol*. **1999**, *6*, 499–503.

(18) Li, L.; Kang, D. G.; Cha, H. J. Functional display of foreign protein on surface of *Escherichia coli* using N-terminal domain of ice nucleation protein. *Biotechnol. Bioeng*. **2004**, *85*, 214–221.

(19) Wu, M. L.; Tsai, C. Y.; Chen, T. H. Cell surface display of Chi92 on *Escherichia coli* using ice nucleation protein for improved catalytic and antifungal activity. *FEMS Microbiol. Lett*. **2006**, *256*, 119–125.

(20) Yim, S. K.; Jung, H. C.; Pan, J. G.; Kang, H. S.; Ahn, T.; Yun, C. H. Functional expression of mammalian NADPH-cytochrome P450 oxidoreductase on the cell surface of *Escherichia coli*. *Protein Expression Purif*. **2006**, *49*, 292–298.

(21) Lan, W. S.; Gu, J. D.; Zhang, J. L.; Shen, B. C.; Jiang, H.; Mulchandani, A.; Chen, W.; Qiao, C. L. Coexpression of two detoxifying pesticide-degrading enzymes in a genetically engineered bacterium. *Int. Biodeterior. Biodegrad*. **2006**, *58*, 70–76.

(22) Yang, C.; Liu, N.; Guo, X. M.; Qiao, C. L. Cloning of *mpd* gene from a chlorpyrifos-degrading bacterium and use of this strain in bioremediation of contaminated soil. *FEMS Microbiol. Lett*. **2006**, *265*, 118–125.

(23) de Lorenzo, V.; Eltis, L.; Kessler, B.; Timmis, K. N. Analysis of *Pseudomonas* gene products using $lacI^q$/*Ptrp-lac* plasmids and transposons that confer conditional phenotypes. *Gene* **1993**, *123*, 17–24.

(24) Liu, Z.; Yang, C.; Jiang, H.; Mulchandani, A.; Chen, W.; Qiao, C. L. Simultaneous degradation of organophosphates and 4-substituted phenols by *Stenotrophomonas* species LZ-1 with surface-displayed organophosphorus hydrolase. *J. Agric. Food Chem*. **2009**, *57*, 6171–6177.

(25) Lei, Y.; Mulchandani, A.; Chen, W. Improved degradation of organophosphorus nerve agents and *p*-nitrophenol by *Pseudomonas putida* JS444 with surface-expressed organophosphorus hydrolase. *Biotechnol. Prog*. **2005**, *21*, 678–681.

(26) Sambrook, J.; Russel, D. W. *Molecular Cloning: A Laboratory Manual*, 3rd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 2001.

(27) Yang, C.; Freudl, R.; Qiao, C. L. Export of methyl parathion hydrolase to the periplasm by the twin-arginine translocation pathway in *Escherichia coli*. *J. Agric. Food Chem*. **2009**, *57*, 8901–8905.

(28) Dumas, D. P.; Caldwell, S. R.; Wild, J. R.; Raushel, F. M. Purification and properties of the phosphotriesterase from *Pseudomonas diminuta*. *J. Biol. Chem*. **1989**, *264*, 19659–19665.

(29) Schmid, D.; Pridmore, D.; Capitani, G.; Battistuta, R.; Nesser, J. R.; Jann, A. Molecular organization of the ice nucleation protein InaV from *Pseudomonas syringae*. *FEBS Lett*. **1997**, *414*, 590–594.

(30) Li, X. H.; He, J.; Li, S. P. Isolation of a chlorpyrifos-degrading bacterium, *Sphingomonas* sp. strain Dsp-2, and cloning of the *mpd* gene. *Res. Microbiol*. **2007**, *158*, 143–149.

(31) Tomlin, C. *The Pesticide Manual*, 13th ed.; BCPC Publications: Hampshire, U.K., 2003.

(32) Horne, I.; Sutherland, T. D.; Harcourt, R. L.; Russell, R. J.; Oakeshott, J. G. Identification of an *opd* (organophosphate degradation) gene in an *Agrobacterium* isolate. *Appl. Environ. Microbiol*. **2002**, *68*, 3371–3376.

(33) Cheng, T.-C.; Harvey, S. P.; Chen, G. L. Cloning and expression of a gene encoding a bacterial enzyme for decontamination of organophosphorus nerve agents and nucleotide sequence of the enzyme. *Appl. Environ. Microbiol*. **1996**, *62*, 1636–1641.

(34) Samuelson, P.; Gunneriusson, E.; Nygren, P. A.; Stahl, S. Display of proteins on bacteria. *J. Biotechnol*. **2002**, *96*, 129–154.

(35) Liu, Z.; Yang, C.; Qiao, C. L. Biodegradation of *p*-nitrophenol and 4-chlorophenol by *Stenotrophomonas* sp. *FEMS Microbiol. Lett*. **2007**, *277*, 150–156.

(36) Ryan, R. P.; Monchy, S.; Cardinale, M.; Taghavi, S.; Crossman, L.; Avison, M. B.; Berg, G.; van der Lelie, D.; Dow, J. M. The versatility and adaptation of bacteria from the genus *Stenotrophomonas*. *Nat. Rev. Microbiol*. **2009**, *7*, 514–525.

Received for review March 23, 2010. Revised manuscript received April 30, 2010. Accepted May 3, 2010. This work was financially supported by the 863 Hi-Tech Research and Development Program of the People's Republic of China (Nos. 2007AA06Z335 and 2007AA061101).