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Simultaneous Degradation of Organophosphates and 4-Substituted Phenols by *Stenotrophomonas* Species LZ-1 with Surface-Displayed Organophosphorus Hydrolase

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Organophosphorous hydrolase (OPH) was expressed onto the surface of a *Stenotrophomonas* species (LZ-1), capable of simultaneously degrading 4-substituted phenols, using the N- and C-terminal domains of ice nucleation protein (INPNC) as an anchoring motif for the first time. The engineered strain LZ-1 could degrade *p*-nitrophenyl-substituted organophosphates as well as their hydrolytic product, PNP, rapidly. Especially, addition of 4-CP (below 0.8 mM) significantly accelerated the complete degradation of above organophosphates (47.1, 34.0, and 40% reduction of time of paraoxon, parathion, and methyl-parathion, respectively) through the accelerated degradation of PNP due to enhanced cell growth supported by 4-CP as the carbon source. OPH could be surface-displayed at a high level without inhibition of cell growth and OPH activity in the presence of 4-CP. In soil samples, strain LZ-1 could also remove these compounds successfully. Functional display of heterologous proteins on the surface of indigenous bacteria could provide a promising technology for effective bioremediation of sites contaminated with mixed organic pollutants.

KEYWORDS: Simultaneous degradation; organophosphates; OPH; ice nucleation protein; surface display; 4-substituted phenols

INTRODUCTION

Organophosphates (OPs) are widely used as pesticides for increasing agricultural products due to their nerve toxicity to inactivate acetylcholinesterase (AChE) in insects. Their acute toxicity to nontarget organisms, including humans, arouses an environmental concern for developing efficient and safe treatment method (*1*).

Compared to the disadvantage of conventional methods for decontamination of pesticides, bioremediation using microorganisms has been considered as a potentially convenient, effective, low-cost, and environmentally friendly method. Organophosphorous hydrolase (OPH), a valuable biological resource for OP decontamination isolated from native soil bacteria *Pseudomonas diminuta* MG and *Flavobacterium* sp., is able to hydrolyze a wide range of OPs such as paraoxon and methyl- and ethyl-parathion effectively (*2*). For overcoming the transport limitation across the outer cell membrane, OPH is targeted onto the cell surface of microorganisms by surface-displaying anchors (*3*). Ice nucleation protein (INP), an outer membrane protein from *Pseudomonas syringae* INA5, is a popular anchor due to its adjustable inframe for passenger proteins with variable molecular weights. The truncated version of INP containing only the N- and C-terminal portions (INPNC) has also been employed for surface targeting (*4*, *5*).

p-Nitrophenol (PNP), the hydrolytic product of *p*-nitrophenyl-substituted OPs, is classified as a priority pollutant by the U.S. EPA (*6*). Genetically engineered bacteria or consortium expressing OPH and PNP degrading enzymes have been used to detoxify those OPs completely (*5*, *7*−*9*).

Organic pollutants usually occur as mixtures in natural waste sites, and the degradation of some compounds in mixtures sometimes is affected, either stimulated or repressed, in the presence of other compounds (*10*−*17*). Similarly, 4-chlorophenol (4-CP), another representive phenolic compound like PNP, is frequently detected in various environments such as wastewater of uncontrolled use of pesticides and herbicides (*18*) and is also listed as a priority pollutant by U.S. EPA (*6*). Bioremediation of environmental pollutants is often limited by coexisting 4-CP due to its high toxicity to microorganisms and cross inhibition effect to the target compounds (*18*−*21*). Therefore, the biodegradation of OPs and their hydrolytic product PNP in the environment might also be inhibited in the presence of 4-CP. To our knowledge, almost nothing is known about the biodegradation behavior of mixtures of OPs and 4-CP. Thus, research on the simultaneous removal of these compounds is of great practical significance for environmental protection.

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We previously isolated a bacterium, identified as *Stenotrophomonas* sp. LZ-1, metabolizing 4-substituted phenols via the hydroquinone pathway exclusively from activated sludge. Mixtures of these phenols were degraded by the strain without cross-inhibition due to the elimination of competition of different catabolic pathways. Furthermore, strain LZ-1 is able to degrade these phenols in soil samples from the environment effectively in a wide range of temperature and pH (*22*).

In this study, the results showed that OPH was successfully displayed on the cell surface of strain LZ-1 using the INPNC anchor. The engineered strain could not only degrade OPs efficiently but also demonstrated an acceleration of the process in the presence of 4-CP. A similar degradation behavior was also observed in soil samples inoculated with the engineered strain. Moreover, a high level of surface expression of OPH did not cause growth inhibition. Due to the above abilities, strain LZ-1 is potentially useful for bioremediation of these pollutants in the environment.

MATERIALS AND METHODS

Chemicals. Paraoxon, parathion, methyl-parathion (analytical grade), and PNP (>99%, HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO). 4-Chlorophenol (>99%, HPLC grade) was obtained from Fluka (Buchs, Switzerland). All other chemicals used were of technical grade.

Bacterial Strains, Plasmids, and Growth Conditions. *Stenotrophomonas* sp. LZ-1 isolated previously from activated sludge by selective enrichment with PNP as the carbon and nitrogen source was used in this study (*22*). Plasmid pPNCO33 containing the INPNC−OPH fusion for surface display was constructed as previously described. Expression of INPNC−OPH was under the control of a tightly regulated *tac* promoter (*5*). To generate plasmid pCPO for expressing OPH in the cytoplasm, the amplified *opd* gene was digested with *Eco*RI and *Hin*dIII and inserted into a similarly digested pPNCO33. The regions encoding OPH without signal sequence were PCR amplified with forward primer 5′-ACGAATTCAGGAAACAATGCAAACGAGAAGG-3′ (the *Eco*RI site is underlined) and reverse primer 5′-ACAAgCTTTCATGACGCCCGCAAGGT-3′ (the *Hin*dIII site is underlined). Plasmid pVLT33 was used as a negative control in experiments.

Strains bearing plasmids were grown in LB medium or minimal salts medium supplemented with kanamycin to a final concentration of 50 μg/mL. Cells were grown in 250 mL flasks at 30 °C and 300 rpm. Transformation of plasmid into *Stenotrophomonas* sp. LZ-1 was done as previously described (*9*, *23*). Expression of the INPNC−OPH fusion was induced with 1 mM IPTG when cells were grown to an OD_{600} = 0.4.

Western Blot Analysis. Cells harboring expression plasmids were harvested after 2 days of incubation, and cell fractionation was performed as Lei et al. described (*9*). Samples of total cell lysate, soluble fraction, and membrane fraction were analyzed on SDS-PAGE with 10% (w/v) acrylamide and electrophoretically transferred onto a nitrocellulose membrane with a tank transfer system. After blocking of nonspecific binding sites with 3% bovine serum albumin (BSA) in Tris-buffered saline with Tween 20 (TBST) solution, the membrane was incubated with rabbit anti-OPH sera (1:1000 dilution) (*3*) at 4 °C overnight and washed with TBST three times. Subsequently, the membrane was allowed to react with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (1:1000 dilution) (Promega, Madison, WI) at 37 °C for 1 h. The membrane was then stained with nitrotetrazolium blue chloride (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Novagen, Darmstadt, Germany) for visualizing antigen−antibody conjugates. Prestained broad-range molecular weight markers were used to estimate protein weights.

Immunofluorescence Microscopy. After 2 days of incubation, cells were harvested and resuspended (OD_{600} = 0.5) in phosphate-buffered saline (PBS) buffer (pH 7.4) with 3% BSA for blocking at 30 °C for 1 h. Intact cells were fixed with polyformaldehyde at 4 °C for 30 min and then incubated with rabbit anti-OPH sera (1:1000) at 4 °C for 8 h. The cells were washed five times and incubated with secondary antibody (goat anti-rabbit IgG) conjugated with fluorescein isothiocyanate (1:100 dilution) (FITC; Sigma) overnight at 4 °C. Cells were washed five times with PBS and observed by a fluorescence microscope (Olympus, Tokyo, Japan). Plasmid pCPO was used as a negative control in experiments.

Proteinase Accessibility Assay. Cells harboring pPNCO33 and pCPO were centrifuged and resuspended in 1 mL of 15% sucrose, 15 mM Tris-HCl, 0.1 mM EDTA, pH 7.8. Samples were incubated for 1 h with 5 μL of 20 mg/mL proteinase K at room temperature. To inhibit further proteinase K activity, 10 μM phenylmethanesulfonyl fluoride was added after incubation. Proteinase K treated and untreated cells were assayed for OPH activity as described above.

Organophosphorus Hydrolase Activity Assay. *Stenotrophomonas* sp. LZ-1 cells harboring pPNCO33 were grown, harvested, and resuspended in 50 mM citrate−phosphate buffer with 50 μM $CoCl_2$ (pH 8.0). Cell lysate was prepared by the addition of 10 μL of lysozyme (10 mg/mL) and incubated on ice for 1 h, followed by sonication in three pulses of 10 s each. Assay mixtures (1 mL, 3% methanol) contain 10 μL of 1.0 OD_{600} cells, 890 μL of 50 mM citrate−phosphate buffer (pH 8.0) with 50 μM $CoCl_2$, and 100 μL of 20 mM paraoxon (added from a 10 mg/mL methanol stock solution). Changes in absorbance (412 nm) were measured for 3 min at 30 °C. Activities were expressed in units (micromoles of paraoxon hydrolyzed per minute per milligram of protein) (e_{412} = 16500 M^{-1} cm^{-1} for *p*-nitrophenol at pH 8.0). Whole cell activity measurements in the presence of 4-CP (1 mM) or not were also conducted similarly.

Stability Study of Resting Culture. Cells containing plasmid pPNCO33 or pPVLT33 were grown in 50 mL of LB medium for 2 days, harvested, washed twice with 50 mM citrate−phosphate buffer (pH 8.0), resuspended in 5 mL of the same buffer with or without 4-CP (1 mM), and incubated at 30 °C. Over the 2 week duration, 0.5 mL of each sample was removed each day. Cell growth and whole-cell OPH activitiy were measured as described above. The influence of different temperatures (20−40 °C) and pH (6−10) on whole-cell OPH activitiy of strain LZ-1 harboring pPNCO33 was also measured.

Degradation of OPs and 4-Substituted Phenols in Pure Cultures by Engineered Strain LZ-1. The *Stenotrophomonas* sp. LZ-1 harboring pPNCO33 was pregrown in LB medium with 50 μg/mL kanamycin overnight at 30 °C and 300 rpm. Subsequently, harvested cells were inoculated (OD_{600} = 0.05) in minimal salts medium (MSM) (*22*) supplemented with 1 mM IPTG, 0.2 mM PNP, 0.1% yeast extract, and 50 μg/mL kanamycin and incubated at 30 °C and 300 rpm until the yellow color of PNP disappeared. The cells were centrifuged at 4 °C, washed with MSM three times, and resuspended in MSM (with 50 μM $CoCl_2$). For OP and phenol degradation tests, 0.4 mM paraoxon, parathion, methyl-parathion, and 4-CP were added to cell suspension (OD_{600} = 0.05). To investigate the effects of initial concentrations of added 4-CP, different initial concentrations of 4-CP (0, 0.4, 0.8, 1.0, and 1.2 mM) were added when the initial OP concentration was fixed. Samples were taken at different time points and diluted with MSM for cell density and substrate concentration analysis. The concentrations of tested compounds were measured as described (*5*, *22*). Cell growth was monitored by measuring the optical density of the culture at 600 nm and was expressed as dry cell weight based on the standard curve. Dry cell weight (mg/L) was found to follow the regression equation X (mg/L) = 742.3(OD_{600}).

Degradation of OPs and 4-Substituted Phenols in Soil Samples by Engineered Strain LZ-1. Soil from the Beijing University, Beijing, China, was used for this study. This soil had characteristics of a sandy loam with 74% sand, 15% silt, 7% clay, 3.97% organic matter, and a pH of 6.7. Soil samples (1 kg) were sterilized by fumigation with chloroform for 10 days at 30 °C. Similar soil samples were stored at 4 °C in sealed polyethylene bags. Residual chloroform was removed from the fumigated soils by repeat evacuation in a vacuum desiccator. Subsamples (100 g) of the fumigated and nonfumigated soils were suspended in 0.1 M phosphate buffer (pH 7.4) containing tested substrates under aseptic conditions (100 mg/kg). One set of fumigated and nonfumigated soils in triplicate was inoculated with engineered strain LZ-1 pregrown in 0.4 mM PNP (10^6 cells/g, quantified by the dilution plate count technique), and another set without inoculation was kept as control. The inoculum was thoroughly mixed into soils under sterile conditions. Soil samples were incubated at 20 °C and 40% of water-holding capacity in the dark. The concentrations of OPs, PNP, and 4-CP were measured as described (*22*, *24*).

Kinetic Modeling. The following equations were used to model the growth and degradation data (*25*):

(1) Microbial exponential growth can be described as $N = N_0 e^{\mu t}$, where N is the concentration of active microorganisms at time t, N_0 is the initial concentration of microorganisms, and μ is the specific growth rate.

(2) The rate of microbial substrate consumption can be described as $-dc/dt = qN_0 e^{\mu t}$, where c is the substrate concentration at time t and q the specific microbial activity.

(3) The remaining concentration of substrate at a certain time is obtained by integrating eq 2: $c = c_0 - qN_0(e^{\mu t} - 1)/\mu$; when $t = 0$, the concentration of substrate equals c_0.

RESULTS

Surface Localization of INPNC−OPH Fusion Protein. The percentage of OPH on the cell surface was estimated by measuring OPH activity in the membrane and soluble cell fractions. Over 90% of the OPH activity was detected in the membrane fractions (**Table 1**). In parallel, more than 90% of OPH activity was present on the cell surface as judged from the activity ratio between whole cells (10.58 units/mg of protein) and cell lysates (11.75 units/mg of protein) (**Table 1**). Protease accessibility experiments were also performed to ascertain the surface localization of OPH. Because proteinase K cannot readily diffuse across the cell membrane, degradation should only occur with proteins exposed on the surface. After 1 h of incubation with proteinase K, the OPH activity for cells carrying pPNCO33 decreased by 86%, whereas cells expressing OPH intracellularly (pCPO) had only a 7% drop in activity.

To test the successful synthesis of INPNC−OPH fusion protein, Western blot analysis was exploited. As shown in **Figure 1**, a major band of approximately 83 kDa, corresponding to the expected molecular mass of INPNC-OPH fusion, was detected from cells harboring pPNCO33. No such signal was detected in controls harboring pVLT33. To assess the distribution of the fusion between the membrane and soluble fractions, total cell lysate, membrane, and soluble fractions were probed with rabbit anti-OPH sera. As shown in **Figure 1**, more than 90% of the fusion was associated with the membrane fraction as judged by the intensity of the protein band, consistent with the activity distribution between the membrane (10.92 units/mg of protein) and soluble fractions (0.72 unit/mg of protein).

To investigate whether the INPNC−OPH fusion was displayed correctly on the bacterial surface in a stable conformation, immunofluorescence microscopy was used. Cells were probed with rabbit anti-OPH sera as a primary antibody and then fluorescently stained with FITC-labeled goat anti-rabbit IgG antibody. As shown in **Figure 2A**, cells harboring pPNCO33 were brightly fluorescent, indicating that OPH was successfully targeted onto the surface. However, no immunostaining was detected in control cells harboring pCPO at all (**Figure 2B**).

OPH Activities. The whole-cell activities of recombinant *Stenotrophomonas* sp. LZ-1 displaying OPH were nearly 60-fold higher than those of the same strain expressing OPH in the cytoplasm when using the same amount of cells. Moreover, strain LZ-1 harboring pPNCO33 exhibited more than 70-fold higher activity than *E. coli* DH5α harboring the same plasmid (*5*). Time course analysis of OPH activity indicated that the highest activity of whole cells appeared at 24 h after induction with 1 mM IPTG (**Figure 3**). However, the highest activity of total cell lysate appeared at 12 h and the activity remained constant thereafter. To investigate the optimal conditions for cell surface-displayed OPH, whole-cell OPH activities of *Stenotrophomonas* sp. LZ-1 harboring pPNCO33 were measured at different temperatures (20−40 °C) and at pH 6−10. The maximum activity was observed at 30 °C and pH 8.0. In the temperature range from 25 to 35 °C and in the pH range from 7 to 9, the whole-cell activities were more than 90% of the maximum activity without significant fluctuation.

Stability Study of the Resting Culture. To test whether continuous surface expression of OPH inhibits cell growth, growth kinetics of cells carrying pPNCO33 and pVLT33 were compared. As shown in **Figure 4A**, both cultures reached the same maximal cell density after 25 h of incubation. No growth inhibition was detected for cells expressing the INPNC−OPH fusion. During the 2 week period, whole-cell activity of LZ-1 harboring the pPNCO33 remained at essentially the original level either in the presence of 4-CP or not (**Figure 4B**).

Degradation of OPs and 4-Substituted Phenols by Engineered Strain LZ-1. As shown in **Figure 5A**, within the first 48 min, paraoxon was rapidly degraded with almost stoichiometric release of PNP. That the degradation pattern of the wild type strain was similar to that of the engineered strain indicated that the surface-expressed OPH did not influence the degradation of PNP (**Figure 5A**). Other tested OPs could also be degraded efficiently. Depletion of parathion and methyl-parathion was observed within 3.5 and 5 h, respectively (**Figure 5B**,**C**). PNP as the hydrolytic product of parathion and methyl-parathion was exhausted within 5 h.

Table 1. Percentage of Surface-Expressed OPH As Estimated from Proteinase K Treatment, Whole Cell versus Lysate Assays, and Membrane Fractionation Experiments[a]

plasmid	% decrease in activity in proteinase K treated cells	whole-cell activity (as % of lysate activity)	% activity in membrane fraction
pPNCO33	86 ± 1.14	90 ± 0.85	93 ± 1.46
pCPO	7 ± 0.57	3 ± 0.36	7 ± 0.79

[a] Experiments were conducted with *Stenotrophomonas* sp. LZ-1 carrying either pPNCO33 or pCPO. The data are the mean ± SD for three independent experiments.

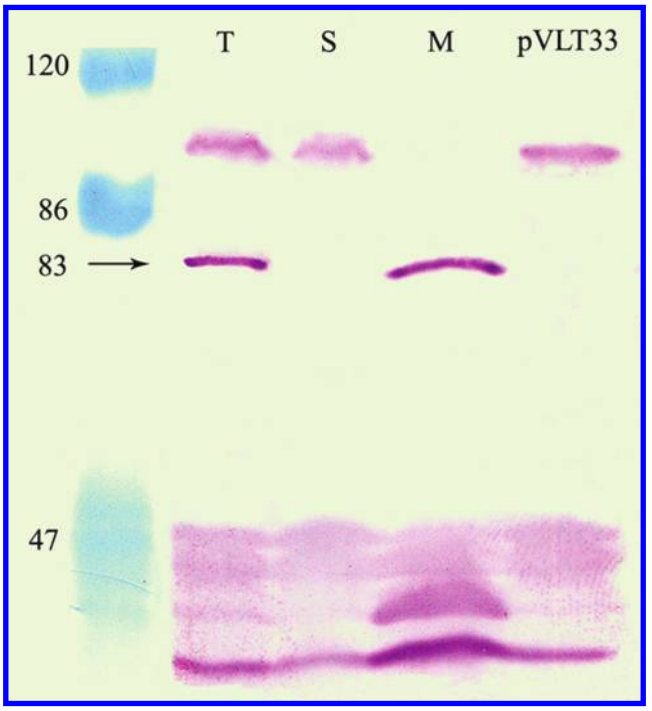

Figure 1. Expression and localization of INPNC−OPH fusion in *Stenotrophomonas* sp. LZ-1 harboring pPNCO33 were assessed by Western blot analysis. The amount of INPNC−OPH in total cell lysate (T), soluble fraction (S), and membrane fraction (M) was probed with rabbit anti-OPH sera at a 1:1000 dilution. Arrow indicates the location of the fusion. Cells harboring pVLT33 were used as a negative control. The molecular mass of marker proteins applied is indicated in kilodaltons.

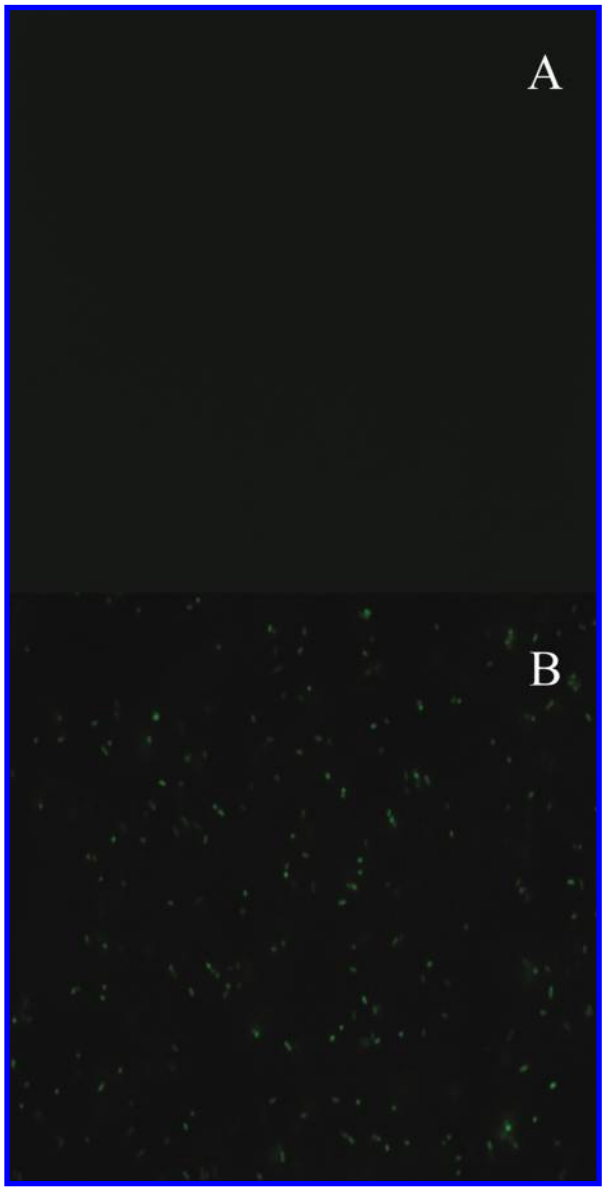

Figure 2. Immunofluorescence micrographs of *Stenotrophomonas* sp. LZ-1 harboring pCPO (**A**) and pPNCO33 (**B**). Cells were probed with rabbit anti-OPH sera and fluorescently stained with goat anti-rabbit IgG−FITC conjugate.

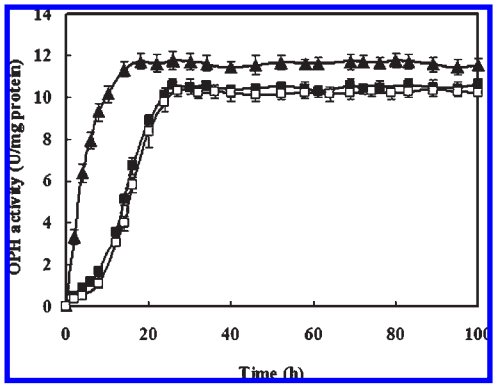

Figure 3. Time course analysis of the activity of OPH surface displaying cells. *Stenotrophomonas* sp. LZ-1 cells were incubated at 30 °C for 40 h after induction with 1 mM IPTG. The activity of whole cell with 1.0 mM 4-CP (■) or not (□) and total cell lysate (▲) was determined as described under Materials and Methods. The data are the mean ± SD for three independent experiments.

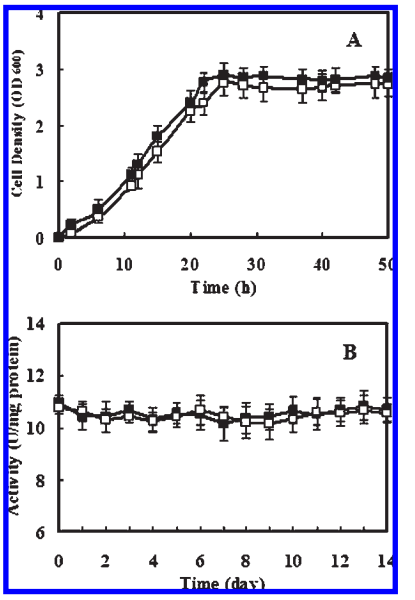

Figure 4. (**A**) Cell growth kinetics of *Stenotrophomonas* sp. LZ-1 harboring pPNCO33 (□) and pVLT33 (■). (**B**) Whole-cell OPH activity in suspended *Stenotrophomonas* sp. LZ-1 culture expressing INPNC−OPH on the surface in the presence of 4-CP (□) or not (■). The data are the mean ± SD for three independent experiments.

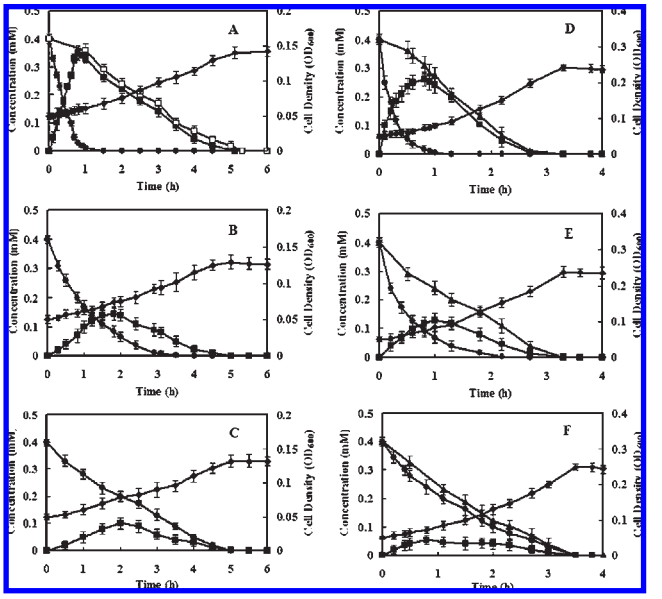

Figure 5. Simultaneous degradation of organophosphates and 4-substituted phenols by *Stenotrophomonas* sp. LZ-1 harboring pPNCO33: (**A**) paraoxon; (**B**) parathion; (**C**) methyl-parathion; (**D**) paraoxon and 4-CP; (**E**) parathion and 4-CP; (**F**) methyl-parathion and 4-CP; (●) organophosphates; (■) PNP formed during the hydrolysis of organophosphates; (▲) 4-chlorophenol; (□) degradation of 0.4 mM PNP by wild type of *Stenotrophomonas* sp. LZ-1; (◆) OD_{600}. All substrates were added at 0.4 mM. The data are the mean ± SD for three independent experiments.

In the presence of 0.4 mM 4-CP, the degradation of paraoxon and 4-CP could be accomplished simultaneously by engineered strain LZ-1 (**Figure 5D**). The addition of 4-CP apparently shortened by 47.1% the time required for the degradation of paraoxon and PNP, whereas the degradation took about 5 h for single paraoxon (**Figure 5A**) compared with less than 3 h for the paraoxon and 4-CP mixture (**Figure 5D**). However, in

Table 2. Mean Values of the Results of Effects of the Addition of 4-CP on the Kinetics of the Degradation of OPs by Strain LZ-1[a]

			specific degradation rate (q) (μmol/h/mg of dry wt)		R^2		
OP (0.4 mM)	4-CP (mM)	growth rate (units h^{-1})	PNP	4-CP	u	q (PNP)	q (4-CP)
paraoxon	0	0.207	1.36	—	0.9882	0.9832	0.9927
	0.4	0.511	1.38	1.31	0.9791	0.9825	0.9973
	0.8	0.579	1.33	1.32	0.9865	0.9896	0.9777
	1.0	0.376	1.25	1.28	0.9944	0.9751	0.9885
	1.2	—	—		—	—	—

[a] The data are the mean for three independent experiments. —, not detected.

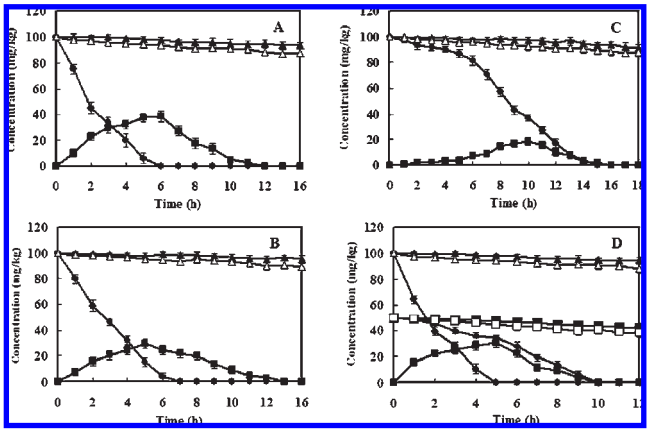

Figure 6. Simultaneous degradation of organophosphates and 4-substituted phenols in soils inoculated with engineered strain LZ-1 at the cell density of 10^6 cells/g: (●) organophosphates in inoculated fumigated soil; (■) PNP in inoculated fumigated soil; (◆) 4-CP in inoculated fumigated soil; (▲) organophosphates in uninoculated fumigated soil; (△) organophosphates in uninoculated unfumigated soil; (■) 4-CP in uninoculated fumigated soil; (□) 4-CP in uninoculated unfumigated soil. The data are the mean ± SD for three independent experiments.

degradation of their mixtures, the rates of initial degradation of PNP were almost unchanged regardless of the change of 4-CP concentrations from 0 to 0.8 mM, and the growth rates of cells in the culture increased apparently compared with that of the degradation of single OPs (**Table 2**). When the initial 4-CP concentration was above 0.8 mM, the growth rate and specific degradation rate of PNP and 4-CP decreased gradually with increasing 4-CP concentration. When the concentration of 4-CP was up to 1.2 mM, degradation of mixtures was almost inhibited (**Table 2**). In the range of 0−1.0 mM 4-CP, the initial hydrolysis rate of OPs remained at a constant level (8.33 μmol/h/mg of dry wt). Similar observation was also found in degradation experiments of parathion and methyl-parathion, and the reductions of degradation time were 34.0 and 40.0%, respectively.

Degradation of OPs and 4-Substituted Phenols in Soil Samples. The inoculation of engineered strain LZ-1 to fumigated and nonfumigated soil samples resulted in an effective degradation of tested OPs. Disappearance of substrates in uninoculated controls was much slower. The addition of 4-CP still accelerated the degradation of OPs. Moreover, the disappearance of substrates in unfumigated controls was slightly more rapid than those in fumigated ones (**Figure 6**). Finally, strain LZ-1 still could be isolated from the soil samples, whereas substrates were removed completely.

DISCUSSION

INPNC is an available surface display system for Gram-negative bacteria, and OPH has been surface displayed by it in hosts that mainly belong to *Escherichia coli* and the *Pseudomonas* genus (*4*, *9*, *23*). To our knowledge, there are almost no similar reports of other bacteria as hosts of INPNC. *Stenotrophomonas* sp., a Gram-negative genus widely distributed in the environment, is a potential genus for bioremediation because its members are able to metabolize a wide variety of xenobiotic compounds such as aromatic and phenolic compounds. Therefore, they have attracted increasing attention in both environmental and biotechnological applications (*26*). However, functional expression of heterologous protein in *Stenotrophmonas* species has not been studied. In this study, the results of Western blot analysis, immunofluorescence microscopy, and proteinase accessibility assay indicated successful surface localization of OPH in strain LZ-1 using INPNC anchor, and the majority of INPNC−OPH fusion proteins were present on the cell surface. The high level of whole-cell activity of engineered strain LZ-1 (10.58 units/mg of protein) that was more than 70-fold higher than that of *E. coli* DH5α using the same anchor (0.14 unit/mg of protein) showed remarkable efficiency of surface expression of OPH in strain LZ-1 (*5*, *24*). The time course of whole-cell activity suggests that stationary-phase translocation of previously synthesized INPNC−OPH fusion may be responsible for the improved whole-cell activity (**Figure 4**) (*5*). Generally, the constitutive expression of outer membrane-linked protein results in growth inhibition and cell lysis that restrict the application of the recombinant bacteria because a high transcription rate could block the translocation pathway (*27*, *28*). In this work, our results of growth kinetics of *Stenotrophomonas* sp. LZ-1 cells carrying pPNCO33 or pVLT33 and of whole-cell activity in a long period showed that no inhibition of growth and cell viability was observed for long-term surface expression of INPNC−OPH fusion, suggesting that INPNC provided stable surface expression of OPH in strain LZ-1. This improvement in surface targeting of OPH in *Stenotrophmonas* sp. might be due to improved membrane translocation because the INPNC anchor originated from *Pseudomonas* strains that were closely related to *Stenotrophmonas* sp. (*29*−*31*). The high efficiency of surface targeting and the remarkable stability of cell growth and whole cell activity in the long term suggested that *Stenotrophomonas* species were potentially appropriate hosts for surface-displaying a wide range of peptides and enzymes. To date, this is the first report of the functional display of heterologous enzymes in a *Stenotrophomonas* species.

In this work, the engineered strain was able to efficiently degrade OPs such as paraoxon, parathion, and methyl-parathion as well as their hydrolytic product PNP. That paraoxon was the most appropriate substrate of OPH was indicated by the pattern of OPH hydrolysis (*2*, *9*).

4-CP is a representative nongrowth substrate of most microorganisms, and it usually exerts cross-inhibition effects on other substrates (*19*, *21*). In this study, we found that the addition of 4-CP shortened time for the degradation of OPs by engineered

strain LZ-1. The accelerated process might be due primarily to the accelerated degradation of PNP as previously reported (*9*). **Table 2** shows the effects of the addition of 4-CP on the kinetics of cell growth and degradation of PNP. The addition of 4-CP supported the cell growth and shortened the time for the degradation of PNP. However, the specific PNP degradation rate did not enhance kinetically. These results suggested that the accelerated PNP degradation was due to the increased cell mass supported by 4-CP as the growth substrate rather than to the improvement of the specific PNP degrading activity of the cells (*10*, *11*). Moreover, no diauxic growth patterns occurred during the simultaneous removal of OPs and 4-CP and the time required for degradation of OPs as well as PNP and 4-CP was the same, indicating that identical enzyme systems were used in PNP and 4-CP degradation (*22*). The effects of initial concentrations of added 4-CP to the degradation of tested OPs were also investigated. When the concentration of 4-CP was low (0−0.8 mM), the degradation of OPs accelerated and the specific degradation rates of PNP and 4-CP had almost no variation. High concentration of 4-CP (> 0.8 mM) did not favor that process, which seems to be due to the toxicity of enhanced concentration of 4-CP to cells. In this study, 4-CP played two roles simultaneously: one was as carbon source to accelerate biodegradation, and the other was as toxic compound to inhibit cell growth. The competition of two effects resulted in the different biodegradation patterns. At the 4-CP concentration of 0.8 mM, the competitive balance was optimal between toxic inhibition and supplies of carbon source, leading to the optimal acceleration of 4-CP biodegradation. Furthermore, the presence of 4-CP did not inhibit whole cell activity in a prolonged cultivation, indicating engineered strain LZ-1 could act steadily in the presence of 4-CP in the long term.

Introducing exogenous microorganisms into polluted sites is an effective approach for accelerating bioremediation (*32*). That engineered strain LZ-1 (10^6 cells/g) could remove OPs and 4-substituted phenols simultaneously in soil samples was indicated by depletion of substrates and isolation of strain LZ-1 after the depletion of substrates. Therefore, the ability of strain LZ-1 with surface-displayed OPH to decontaminate those pollutants in soils was proved preliminarily.

All of the above results highlight that engineered LZ-1 is a promising whole-cell biocatalyst for satisfying the requirements of steady bioremediation of OPs and 4-substituted phenols in the natural environment. To our knowledge, this is the first report of the simultaneous degradation of OPs and 4-CP.

In future work, other suitable surface-displaying anchors and heterologous proteins for strain LZ-1 would be tested.

Supporting Information Available: The support information is about an illustration of the structures of the compounds being studied in this work. This material is available free of charge via the Internet at http://pubs.acs.org.

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