

Simultaneous Degradation of Organophosphorus Pesticides and *p*-Nitrophenol by a Genetically Engineered *Moraxella* sp. with Surface-Expressed Organophosphorus Hydrolase

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Abstract: *Moraxella* sp., a native soil organism that grows on *p*-nitrophenol (PNP), was genetically engineered for the simultaneous degradation of organophosphorus (OP) pesticides and *p*-nitrophenol (PNP). The truncated ice nucleation protein (INPNC) anchor was used to target the pesticide-hydrolyzing enzyme, organophosphorus hydrolase (OPH), onto the surface of *Moraxella* sp., alleviating the potential substrate uptake limitation. A shuttle vector, pPNCO33, coding for INPNC-OPH was constructed and the translocation, surface display, and functionality of OPH were demonstrated in both *E. coli* and *Moraxella* sp. However, whole cell activity was 70-fold higher in *Moraxella* sp. than *E. coli*. The resulting *Moraxella* sp. degraded organophosphates as well as PNP rapidly, all within 10 h. The initial hydrolysis rate was 0.6 $\mu\text{mol/h/mg}$ dry weight, 1.5 $\mu\text{mol/h/mg}$ dry weight, and 9.0 $\mu\text{mol/h/mg}$ dry weight for methyl parathion, parathion, and paraoxon, respectively. The possibility of rapidly degrading OP pesticides and their byproducts should open up new opportunities for improved remediation of OP nerve agents in the future. © 2001 John Wiley & Sons, Inc. *Biotechnol Bioeng* 76: 318–324, 2001.

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INTRODUCTION

As the demand for agricultural produce increases, so inevitably does the need for pesticides. Currently, organophosphate compounds are one of the most widely used classes of pesticide in industrialized countries. In the United States, over 40 million kilograms of or-

ganophosphate pesticides are applied annually (Mulchandani et al., 1999). High-level exposure to these neurotoxins results in acetylcholine accumulation, which interferes with muscular responses, leading to the possibility of death. Repeated or prolonged exposure can cause delayed cholinergic toxicity and neurotoxicity (Tuovinen et al., 1994).

Parathion and methyl parathion are two popular organophosphorus pesticides used for agriculture crop protection (Kumar et al., 1996). Because of their widespread usage, there is a need to detoxify the large quantities of wastes generated including: (1) residual (i.e., excess or unused) pesticide concentrates remaining in their original containers; and (2) dilute aqueous pesticide solutions generated by pesticide producers and by consumers who wash storage tanks and spray equipment (Munnecke, 1979).

Organophosphorus hydrolase (OPH) isolated from soil microorganisms has been shown to hydrolyze a wide range of organophosphate pesticides (Mulbry and Karns, 1988; Serdar et al., 1989). Enzymatic hydrolysis of parathion and methyl parathion reduces the toxicity by nearly 120-fold (Munnecke, 1979) and leads to the formation of dialkylthiophosphates and *p*-nitrophenol (PNP) as byproducts (Munnecke and Hsieh, 1976). Although these byproducts are considerably less toxic, their fate in the environment is of potential concern. Although the toxicity of dialkylthiophosphates is not well characterized, microorganisms that utilize them as sole phosphorus sources have been isolated (Cook et al., 1978). On the other hand, the toxic effect of PNP on biological systems has led to its classification as a priority pollutant by the U.S. EPA (Errampalli et al., 1999). Unlike organophosphates, which are not water-soluble, PNP is highly water-soluble and is found in both terrestrial and aquatic environments (Zaidi and Imam, 1996). Different bacteria have been

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shown to grow on parathion and methyl parathion; however, the rate of degradation is usually slow and complete mineralization may require up to 2 days (Rani and Lalithakumari, 1994; Sidderamappa et al., 1973).

Bacterial strains have been isolated for their ability to degrade PNP (Leung et al., 1997; Nishino and Spain, 1993; Spain et al., 1979). A *Moraxella* sp. isolated from activated sludge utilizes PNP as the sole carbon source (Spain and Gibson, 1991). PNP is degraded by an initial monooxygenase attack with the concomitant release of nitrite and the production of hydroquinone. Degradation of hydroquinone proceeds by ring-fission to form γ -hydroxymuconic semialdehyde, which is subsequently converted to maleylacetate by a dehydrogenase. Maleylacetate is then further reduced to β -keto adipate and TCA cycle intermediates.

Because *Moraxella* sp. can rapidly degrade PNP, concomitant expression of OPH would result in a recombinant strain capable of rapidly degrading organophosphates as well as PNP. The use of *Moraxella* sp. for in situ degradation is advantageous because they are known to survive in contaminated environments for an extended period of time (Tresse et al., 1998). Because the transport of these pesticides across the cell membrane has been shown to be restrictive (Elashvili et al., 1998; Hung and Liao, 1996), one solution is to target OPH directly on the surface of *Moraxella* sp. (Richins et al., 1997). Although various surface expression systems have been developed for Gram-negative bacteria (Charbit et al., 1986; Francisco et al., 1992; Fuchs et al., 1991; Klauser et al., 1990), there are no reports of the functional expression of enzymes on the surface of any Gram-negative bacteria other than *E. coli*.

Ice nucleation protein (INP), an outer membrane protein from *Pseudomonas syringae*, is composed of three distinct domains: an N-terminal domain (15%) containing three or four potential transmembrane spans; a C-terminal domain (4%); and a central domain composed of repeating residues, which acts as the template for ice nucleation (Wolber, 1993). Recently, INP from *P. syringae* KCTC1832 (InaK) has been used as an anchoring protein for the cell surface expression of levansucrase and methylcarboxycellulase (Jung et al., 1998a, 1998b). Even the truncated version of INP containing only the N- and C-terminal portion (INPNC) was shown to be sufficient for targeting proteins to the cell surface of *E. coli* (Jung et al., 1998a) and *Salmonella* (Lee et al., 2000). Other related INPs have also been used for cell surface expression (Bassi et al., 2000; Shimazu et al., 2001).

In this work, we demonstrate the functional expression of OPH on the surface of *Moraxella* sp., a natural PNP degrader, using the INPNC anchor. The result is a single microorganism that is endowed with the capability to rapidly degrade organophosphate pesticides and PNP simultaneously.

EXPERIMENTAL

Bacterial Strains and Plasmids

Escherichia coli strain XL1-Blue (*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac* [F' , *proAB*, *lacI^q*Z Δ M15, Tn10 (Tet^r)] and a *Moraxella* sp. isolated from activated sludge by selective enrichment with PNP (Spain and Gibson, 1991) were used in this study. Plasmid pDP245 (Schmid et al., 1997) was used as template for the *inaV* gene. Plasmid pCOPH-C1 (Richins et al., 2000), carrying a hybrid *cbd-opd* gene, was used as the source of the *opd* gene. A shuttle vector, pVLT33 (de Lorenzo et al., 1993), was used for expression of INPNC-OPH fusions.

Strains bearing plasmids were grown in Luria-Bertani (LB) media supplemented with kanamycin to a final concentration of 50 μ g/mL. Cells were grown in 250-mL flasks in an Innova 4000 shaker (New Brunswick Scientific) with vigorous agitation (300 rpm) at either 25°C or 30°C. Bacteria harboring expression vectors were grown to an OD₆₀₀ = 0.4 before induction with 1 mM IPTG.

Construction of INPNC-OPH Fusion

Truncated InaV OPH fusion was constructed as follows. PCR was used to clone the N- and C-terminal domains using plasmid pDP245 as template. Primers 5'-GGGAATTCAGGAAACAATGAATATCGACAAA-GCGTTGGTA-3' and 5'-CCGGTACCAATCAGATCACTGTGGTTGCCAGC-3' were used to amplify the N-terminal region, and primers 5'-CCGGATCCTTCTCGACCTCTATCCAGTC-3' and 5'-CCGGTACCGGCGGGCATGGCTAGCACG-3' were used for the C-terminal region. The resulting 500-bp N-terminal fragment was digested with *EcoRI* and *KpnI* and ligated into a similarly digested pUC18 resulting in pIVN. The 300-bp C-terminal domain was *KpnI*/*BamHI* digested and ligated into pIVN to give pIVNC. To create an in-frame INPNC-OPH fusion, a 1.1-kb fragment of the *opd* gene was *BamHI* and *HindIII* digested from pCOPH-C1 and inserted into similarly digested pIVNC to generate pINCOP. The *inpnc-opd* fragment was released from pINCOP with *EcoRI*-*HindIII* and the resulting 2-kb fragment was subcloned into an *E. coli*/*Pseudomonas* shuttle vector, pVLT33, to create pPNCO33. Expression of INPNC-OPH was under the control of a tightly regulated *tac* promoter.

Organophosphorus Hydrolase Assay

Induced cultures were grown for 2 days at 25°C or 30°C and harvested. Cells were resuspended in 50 mM citrate-phosphate buffer with 50 μ M CoCl₂ (pH 8.0). The spectrophotometric assay was conducted in a 1.5-mL disposable methacrylate cuvette (Fisher, Tustin, CA). Cell lysate was prepared by addition of 10 μ L of lysozyme

(10 mg/mL) and incubated on ice for 1 h, followed by sonication for 15 seconds. For each assay, 10 μ L of cells ($OD_{600}=1.0$) was added to 890 μ L citrate-phosphate buffer and 100 μ L of 20 mM paraoxon (Sigma). Methanol was added to a final concentration of 10%. Changes in absorbance (410 nm) were measured for 2 min at 37°C. Activities were expressed as in units (micromoles of paraoxon hydrolyzed per minute), per milligram of protein (ϵ_{410} 16,500 M^{-1} cm^{-1} for *p*-nitrophenol).

Immunofluorescence Microscopy

Following 2-day incubation, cells were harvested and resuspended ($OD_{600}=0.5$) in phosphate buffered saline (PBS) with 3% bovine serum albumin (BSA). Intact cells were then incubated with rabbit anti-OPH antisera (1:3000) (Richins et al., 2000) for 8 h at 4°C. The cells were washed extensively, resuspended in PBS with secondary antibody goat anti-rabbit immunoglobulin (IgG) conjugated with fluorescein isothiocyanate (FITC; Sigma) at a dilution 1:64, and incubated overnight at 4°C. Prior to microscopy, cells were washed five times with PBS. Photographs were taken using an immunofluorescence microscope (Nikon).

Cell Fractionation

After 24 and 48 h of growth, cells were harvested and resuspended in 25 mM Tris-Cl buffer (pH 8.0). Cells were disrupted by a French pressure cell at 16,000 psi (SLM Instruments, Inc.). After two passes through the French Press, cells were centrifuged for 10 min at 10,000 rpm to remove cell debris. The cell free extract was then centrifuged at 115,000g (Beckman) to separate the membrane and soluble fractions. The membrane fraction was resuspended in equal amount of 25 mM Tris-Cl buffer (pH 8.0). Ten microliters of total lysate, membrane, and soluble fractions were used for western blot analysis.

Western Blot Analysis

Samples (10 μ L) of 2.5 \times concentrated cells were mixed with loading buffer (Laemmli, 1970) and boiled for 10 min. Samples were run using sodium dodecylsulfate 10% (w/v) polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to a nitrocellulose support before incubation with OPH (Richins et al., 2000) or INPNC (Kwark et al., 1999) specific antisera. Western blot analysis was performed using an Immun-Blot GAR-AP kit (BioRad, Hercules, CA). Prestained broad-range molecular-weight markers were used to estimate protein weights.

Organophosphates/PNP Degradation

For the OPH/PNP degradation experiments, *Moraxella* harboring plasmid pPNCO33 was inoculated at an

$OD_{600} = 0.05$ in media containing 0.1% yeast extract supplemented with M9 salts and incubated at 30°C. Cells were induced with 1 mM IPTG at an $OD_{600} = 0.2$. Immediately following induction, 0.4 mM PNP was added as the carbon source. After all the PNP was degraded, 0.4 mM parathion, methyl parathion, or paraoxon was added to the cell suspension ($OD_{600} = 0.4$). Methanol was also added to a final concentration of 10% to increase solubility. One-hundred-microliters of samples were taken at various timepoints, diluted with 900 μ L of water, and monitored spectrophotometrically for PNP formation. To determine the residual pesticide concentration, 100- μ L samples were taken at various timepoints, centrifuged to remove the cells, and combined with 10 μ L of crude extract from fractionated cells. The reaction was allowed to proceed until all unreacted paraoxon, parathion, or methyl parathion was hydrolyzed, and the residual concentration of pesticide was calculated from amount of *p*-nitrophenol formed (Mulchandani et al., 2000).

RESULTS

Surface Expression of OPH Using Ice Nucleation Protein

To investigate the feasibility of targeting OPH to the surface of *Moraxella* sp., the truncated InaV (INPNC) protein from *Pseudomonas syringae* INA5 was used as the surface anchor. Plasmid pPNCO33, carrying the *inpnc-opd* fusion was constructed by inserting the *inpnc-opd* fragment into a shuttle vector, pVLT33. Expression of the INPNC-OPH fusion was under control of a *tac* promoter. *E. coli* and *Moraxella* sp. harboring pPNCO33 were grown at 30°C and the production of INPNC-OPH was induced with IPTG. Whole-cell activity was more than 70-fold higher in *Moraxella* sp. (9.85 U/mg protein) than *E. coli* (0.14 U/mg protein). This is also nearly 80-fold higher than the value obtained from *E. coli* expressing OPH on the cell surface using the Lpp-OmpA fusion (Kaneva et al., 1998).

Induced cultures of *Moraxella* sp. were viable and fully active during prolonged incubation for 48 h. Production of full-size INPNC-OPH fusions was probed with both OPH and INPNC antisera (Fig. 1). A major band of approximately 85 kDa was detected from cells carrying pPNCO33, whereas no such protein was detected with control cells carrying pVLT33. A minor band of 35 kDa was also detected with the OPH antisera, suggesting that OPH may be released from the fusions by limited proteolysis. The expression level of INPNC-OPH fusions reached a maximum at 24 h and remained constant thereafter (Fig. 1B). However, whole cell activity was nearly 60-fold higher for the 48-h cultures (Table I). The same difference in OPH activity was detected with total cell lysates. This suggests that

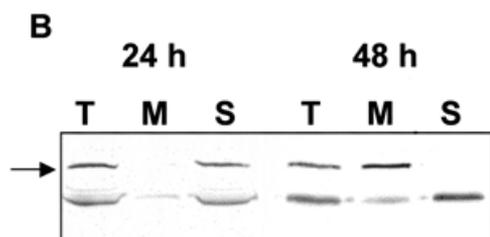
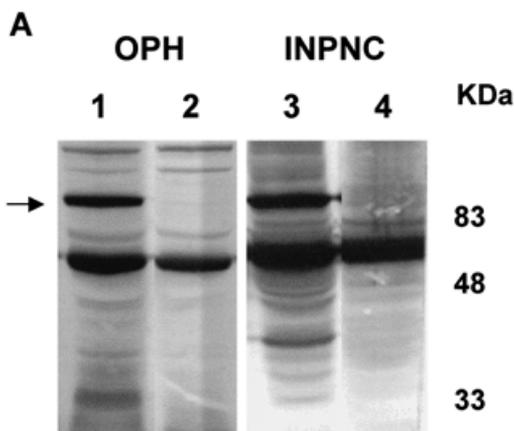


Figure 1. Expression and localization of INPNC–OPH fusions. (A) Western blot analysis with OPH antibody or INPNC antibody. Whole cell lysates of *Moraxella* sp. harboring pPNCO33 (1, 3) or pVLT33 (2, 4) were used. (B) Western blot analysis with total cell lysates (T), membrane fractions (M), and soluble fractions (S) from 24- and 48-h cultures. OPH antibodies were used at a 1:5000 dilution.

Table I. Percentage of surface-exposed OPH as estimated from whole cell versus lysate assays.^a

	OPH activity (U/mg protein)	
	Whole cell	Cell lysate
24 h	0.161	0.262
48 h	9.85	9.90

^aExperiments conducted with *Moraxella* sp. carrying pPNCO33.

stationary-phase translocation or processing of previously synthesized OPH fusions may be responsible for the improved whole cell activity. It is possible that only properly translocated OPH fusions could retain functionality. A similar observation has been reported for the surface expression of OPH using Lpp–OpmA fusion (Kaneva et al., 1998). To test this hypothesis, the membrane and soluble fractions from 24- and 48-h cultures were subjected to immunoblotting with OPH antisera (Fig. 1B) and OPH assay (Table I). For 24-h cultures, the majority of INPNC–OPH and activity was detected in the soluble fraction. In contrast, most of the OPH fusions and activity were detected in the membrane fraction for the 48-h cultures. This result confirmed that INPNC–OPH fusions are active only when properly translocated.

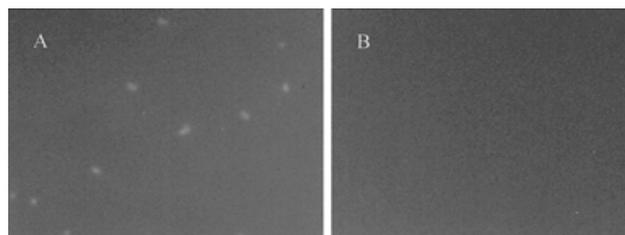


Figure 2. Immunofluorescence micrographs of *Moraxella* sp. harboring (A) pPNCO33 and (B) pVLT33. Cells were probed with anti-OPH antisera and fluorescently stained with goat anti-rabbit IgG–FITC conjugate.

Surface Localization of OPH in *Moraxella* sp.

To ascertain the presence of enzymatically active OPH on the cell surface, the rates of paraoxon degradation by intact cells were compared to their corresponding cell lysates. In parallel, more than 95% of OPH activity was present on the cell surface in the 48-h samples, as judged from the activity ratio between whole cells and cell lysates (Table I). This result agrees well with the sub-cellular localization of the INPNC–OPH fusions. In contrast to previous results with INP fusions (Jung et al., 1998a; Lee et al., 2000), whole cell activities as well as the extent of surface expression were the same at both 25°C and 30°C.

To clearly demonstrate the surface localization of OPH, immunofluorescence microscopy was used. Cells were probed with rabbit anti-OPH serum as a primary antibody and then fluorescently stained with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG antibody. As shown in Figure 2A, cells harboring pPNCO33 were brightly fluorescent, indicating that OPH was successfully displayed on the surface. Cells carrying only pVLT33 were not stained at all with the FITC-labeled secondary antibody (Fig. 2B). From these results, we concluded that OPH was indeed functionally displayed on the cell surface using the INPNC anchor.

Simultaneous Degradation of Organophosphates and PNP

To investigate whether the expression of OPH on the surface enabled *Moraxella* sp. to simultaneously degrade organophosphate pesticides and PNP, cultures of *Moraxella* sp. carrying pPNCO33 were first grown in M9 medium with PNP as the sole carbon source. After all PNP was degraded, paraoxon was added to final concentration of 0.4 mM. As depicted in Figure 3A, paraoxon was quickly degraded within the first 40 min with almost stoichiometric release of PNP. PNP degradation (0.6 $\mu\text{mol/h/mg}$ dry weight) occurred on a slower time scale compared with the initial hydrolysis of paraoxon (9.0 $\mu\text{mol/h/mg}$ dry weight) with all PNP de-

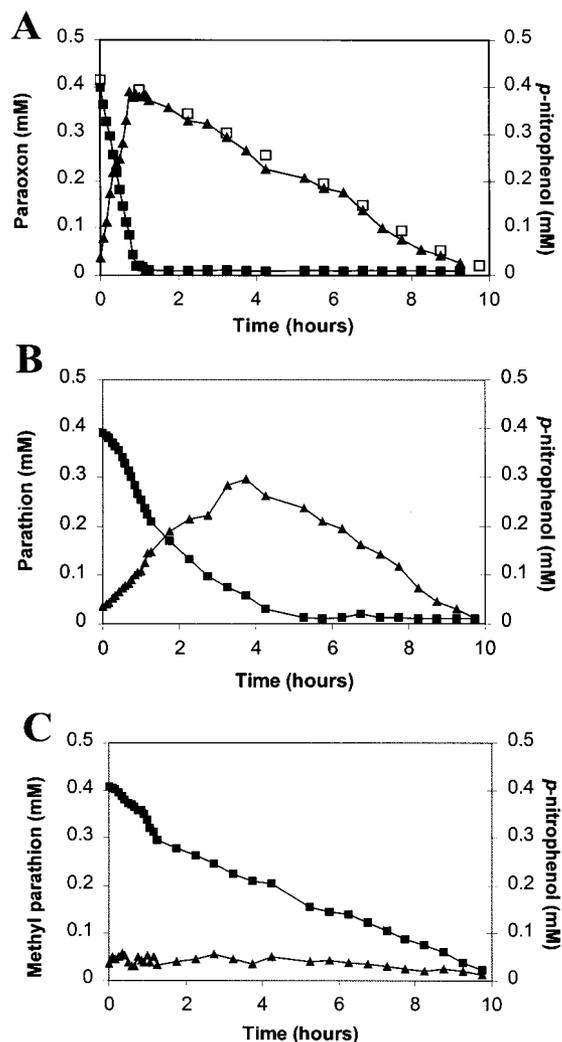


Figure 3. Simultaneous degradation of (A) paraoxon, (B) parathion, or (C) methyl parathion and PNP by *Moraxella* sp. harboring pNCO33. All substrates were added at an initial concentration of 0.4 mM. Symbols: (■) organophosphorus pesticide; (▲) PNP. Degradation of 0.4 mM PNP (□) by wild-type *Moraxella* sp. also shown in (A).

graded within 10 h. The presence of OPH on the cell surface had no impact on PNP degradation; wild-type *Moraxella* sp. degraded PNP at the same rate as the recombinant strains (Fig. 3A).

To demonstrate that other pesticides can be similarly degraded, the degradation of 0.4 mM parathion and methyl parathion was tested. Complete hydrolysis of parathion and methyl parathion occurred within 4 h (1.5 $\mu\text{mol/h/mg}$ dry weight) and 10 h (0.6 $\mu\text{mol/h/mg}$ dry weight), respectively (Fig. 3B, C). This reduction in hydrolysis rate is consistent with the kinetic properties of OPH, which is 5 to 40 times more efficient in hydrolyzing paraoxon than parathion and methyl parathion (Dumas et al., 1993). Again, the PNP produced from hydrolysis was completely degraded within 10 h. For methyl parathion, the rate of hydrolysis was similar

to the rate of PNP degradation, resulting in only low-level accumulation of PNP.

DISCUSSION

Parathion and methyl parathion are highly toxic organophosphorus nerve agents that are commonly used as insecticides. Microorganisms that can rapidly mineralize these pesticides have not yet been isolated. Fortunately, a diverse set of organisms has been isolated that are capable of collectively mineralizing these pesticides. Some species can hydrolyze parathion rapidly but produce PNP as one of the by products. Others can take PNP and use it as the sole carbon source. By recruiting the desirable characteristics from various species, it is possible to engineer a single organism that can rapidly degrade both organophosphate pesticides and PNP.

To confer the capability to simultaneously degrade organophosphorus pesticides and PNP on a natural PNP degrader, *Moraxella* sp., the gene coding for OPH was introduced. The production of OPH on the cell surface, rather than intracellularly, has been shown to offer a more effective way of hydrolyzing organophosphates (Richins et al., 1997). Although there were a few examples of expressing peptides and small antigens on the surface of *Pseudomonas* or *Salmonella* (Cebolla et al., 1996; Lee et al., 2000; Valls et al., 2000), the expression of functional enzymes on the surface of Gram-negative bacteria other than *E. coli* has never been reported. The initial challenge was to develop an efficient system to express functional OPH on the surface of *Moraxella* sp. Not only were we able to accomplish this using the truncated ice nucleation protein anchor, the efficiency of surface expression was 70-fold higher in *Moraxella* sp. than in *E. coli*. It should be noted that the truncated InaV protein from *Pseudomonas syringae* INA5 (Schmid et al., 1997) was used in this study rather than the previously described InaK protein from *P. syringae* KCTC1832 (Jung et al., 1998). The rationale was based on our previous results showing improved OPH activity using the InaV anchor in *E. coli* (Shimazu et al., 2001). In fact, the use of InaK anchor resulted in 100-fold less activity in *Moraxella* sp. (data not shown).

Moraxella sp. with surface-expressed OPH degraded organophosphorus pesticides rapidly. The initial hydrolysis rate of 0.6 and 1.5 $\mu\text{mol/h/mg}$ dry weight cell for methyl parathion and parathion, respectively, are about tenfold faster than the rate of 0.04 and 0.14 $\mu\text{mol/h/mg}$ dry weight cell (Siddaramappa et al., 1973) previously reported for the same two substrates by other species. In fact, the rate of degradation appears to be limited by the kinetic properties of OPH, which is five times more efficient in hydrolyzing parathion than methyl parathion. To enhance the overall degradation of less efficient substrates such as methyl parathion, enzymes with improved hydrolytic activity must be de-

veloped. Recent success with directed evolution has provided the feasibility toward this end (Bruhlmann and Chen, 1999). For substrates that are quickly hydrolyzed, such as paraoxon or parathion, PNP degradation became rate-limiting, resulting in the net accumulation of PNP. Because the native pathway of PNP degradation is induced in the presence of PNP, it may be possible to further increase the rate of PNP degradation by over-expressing the entire pathway using a constitutive promoter.

Moraxella sp. was capable of targeting the INPNC-OPH fusion 70-fold more efficiently compared to commonly used *E. coli*. This remarkably high level of surface expression could potentially be utilized for the display of a wide range of peptides and enzymes onto the surface of *Moraxella* sp. These strategies are currently under investigation.

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