

Altering the Substrate Specificity of Organophosphorus Hydrolase for Enhanced Hydrolysis of Chlorpyrifos

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Received 23 April 2004/Accepted 27 April 2004

Chlorpyrifos is one of the most popular pesticides used for agriculture crop protection, and widespread contamination is a potential concern. However, chlorpyrifos is hydrolyzed almost 1,000-fold slower than the preferred substrate, paraoxon, by organophosphorus hydrolase (OPH), an enzyme that can degrade a broad range of organophosphate pesticides. We have recently demonstrated that directed evolution can be used to generate OPH variants with up to 25-fold improvement in hydrolysis of methyl parathion. The obvious question and challenge are whether similar success could be achieved with this poorly hydrolyzed substrate, chlorpyrifos. For this study, five improved variants were selected from two rounds of directed evolution based on the formation of clear halos on Luria-Bertani plates overlaid with chlorpyrifos. One variant, B3561, exhibited a 725-fold increase in the k_{cat}/K_m value for chlorpyrifos hydrolysis as well as enhanced hydrolysis rates for several other OP compounds tested. Considering that wild-type OPH hydrolyzes paraoxon at a rate close to the diffusion control limit, the 39-fold improvement in hydrolysis of paraoxon by B3561 suggests that this variant is one of the most efficient enzymes available to attack a wide spectrum of organophosphate nerve agents.

Organophosphorus (OP) compounds are highly toxic because they inhibit acetylcholine esterase in the central nervous system synapses, leading to a subsequent loss of nerve function and eventual death (5). Increasing concern about national security has focused our attention on these highly toxic agents because of their potential threat as nerve agents for chemical warfare and their widespread availability as agricultural insecticides (8). Novel methods for their sensitive detection and rapid decontamination are needed to respond to emergency situations.

Organophosphorus hydrolase (OPH), an enzyme isolated from *Pseudomonas diminuta* MG and *Flavobacterium* sp. strain ATCC 27551, has attracted considerable interest because of its ability to hydrolyze different insecticides as well as the more toxic chemical-warfare agents, such as soman and VX (6). Hydrolysis of OP compounds by OPH dramatically reduces their toxicity (4), providing a promising enzymatic decontamination technology. Unfortunately, the rates of hydrolysis by OPH differ dramatically for members of the family of OP compounds, ranging from hydrolysis at the diffusion-controlled limit for paraoxon to several orders of magnitude slower for malathion, chlorpyrifos, and VX (6, 9). Although site-directed mutagenesis has been used to improve the substrate specificity and stereoselectivity of OPH (4, 12, 13, 18, 19), our ability to deduce substitutions that are important for substrate specificity is still limited to the active-site residues.

Directed evolution has recently been used to generate OPH variants with up to 25-fold improvements in hydrolysis of methyl parathion (3), a substrate that is hydrolyzed 30-fold less efficiently than paraoxon. Since only a few mutations were

shown to play key roles in enhancing the overall catalytic efficiency, there is a realistic possibility to create a cocktail of OPH variants that can be rationally combined for rapid decontamination and detection of OP nerve agents. The obvious question is whether similar success could be achieved with other poorly hydrolyzed substrates.

In this paper, we describe the directed evolution of OPH to improve the hydrolysis of a poorly hydrolyzable substrate, chlorpyrifos (~1,200-fold less efficient than paraoxon) (1). Up to 700-fold improvement was obtained, and the best variant hydrolyzes chlorpyrifos at a rate similar to that of the hydrolysis of paraoxon by wild-type OPH. This result suggests that directed evolution of OPH and related enzymes may lead to potential biological solutions for efficient decontamination and detection of neurotoxic nerve agents, such as sarin and VX.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* strain XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17* [r_{K}^{-} m_{K}^{+}] *supE44 relA1 lac* [F' *proAB lacI*^qZAM15 Tn10 Tet^r]) was used in all experiments. Plasmid pINCOP (14) harboring the INP-OPH fusion was used to display OPH variants on the cell surface of *E. coli*. Plasmid pPROEXHTa (GIBCO BRL) was used for intracellular expression of wild-type OPH and the variants as an N-terminal fusion to a hexahistidine tag (His₆) for easy purification (3).

DNA shuffling and library construction. The wild-type *opd* gene and the best variant, 22A11, obtained from improved degradation of methyl parathion (3), were used as templates for DNA shuffling (17). Primers IB655 (5'-GGCGGGA GACCAAAGCAGATT-3') and DH100 (5'-CCCCAGGCTTTACTTTTAT-3') were used to amplify a 1.3-kb *opd* fragment. Following purification by using the Wizard PCR purification kit (Promega, Madison, Wis.), the PCR fragments were digested with 0.01 U of DNase I (Boehringer Mannheim) at 15°C for 8 min. The reaction was stopped by heating at 90°C for 10 min. DNA fragments of fewer than 60 bp were isolated from a 2% agarose gel with a DEAE-cellulose membrane (Schleicher & Schuell). Approximately 1.5 μg of the two fragmented templates was mixed together and reassembled in a 100-μl volume of primerless PCR by using the EasyStart (Molecular Bio-Products) mix and *Taq* DNA polymerase (Promega). Conditions for PCR were as follows: 5 min at 94°C and 80 cycles of 30 s at 94°C, 10 s at 52°C, and 10 s at 72°C, followed by 2 min at 72°C. The reassembled product was diluted 40 times and used in an additional PCR

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amplification using the M13/pUC sequencing primer and the IB778 primer (5'-CACCATCGGCACTCGTCCGTC-3'). The 1.2-kb amplification product was recovered using a Gene Clean II kit (QBIOSYSTEMS), digested with BamHI and HindIII, and subcloned into a similarly digested pINCOP to generate a library of OPH variants fused to the ice nucleation protein (INP) anchor. The resulting plasmids were used to transform *E. coli* XL1-Blue by the scanning electron microscopy method (10).

Screening of OPH variants for improved hydrolysis of chlorpyrifos. A simple plate assay based on the formation of clear haloes as a result of chlorpyrifos hydrolysis was developed for rapid prescreening of improved OPH variants. Transformants were streaked onto Luria-Bertani plates containing 0.75 mM chlorpyrifos and 100 µg of ampicillin/ml and maintained at 30°C for 48 h. Colonies were identified based on the formation of a larger clearing zone on the plate. Potential clones were tested again using a top agar plate assay. Single colonies were restreaked onto Luria-Bertani plates containing 100 µg of ampicillin/ml and 10 µM CoCl₂ and maintained for 45 h. A 0.7% agar solution containing 50 mM phosphate-citrate buffer (pH 8.0) and 0.75 mM chlorpyrifos was overlaid. After 2 to 20 h of incubation at 37°C, the size of the halo formed was evaluated to confirm the improvement of the hydrolysis rate for the potential variant.

DNA sequencing. Variants were amplified in both forward and reverse directions using an automated dye terminator kit including fluorophore-labeled dideoxynucleotide triphosphates. The extension products were fractionated by capillary electrophoresis and analyzed by an Applied Biosystems model 3100 DNA sequencer with an 80-cm array. Small-scale isolation of plasmid DNA for sequencing was carried out using a Wizard Plus Minipreps DNA purification system (Promega).

Purification of variants. To determine the kinetic properties of the OPH variants, the *opd* genes were excised with BamHI and HindIII and ligated into pPROEXHTa (GIBCO BRL) to generate the plasmid pHOP. This plasmid allowed the intracellular expression of the OPH variants as an N-terminal fusion to a hexahistidine tag (His₆). All variants were purified according to the procedure described previously (3).

OPH assay. Purified variants were resuspended in 1 ml of 50 mM CHES buffer (pH 9.0) containing 0.2 mM chlorpyrifos, 10% methanol, 2.5% polyethylene glycol 8000, and 0.1% polyoxyethylene-10 lauryl ether (Sigma) in 1.5-ml disposable methacrylate cuvettes (Fisher). Hydrolysis of chlorpyrifos was measured by changes in absorbance at 276 nm ($\epsilon_{276} = 2,790 \text{ M}^{-1} \text{ cm}^{-1}$ for chlorpyrifos) using a Beckman model DU-60 spectrophotometer for 5 min at 37°C. All assays were performed in triplicate. Enzyme concentrations were determined according to the Bradford method (Bio-Rad) using bovine serum albumin as the standard. Specific activities were expressed as units (micromoles of chlorpyrifos hydrolyzed per minute) per milligram of protein.

The kinetic constants for each variant were obtained by measuring the initial hydrolysis rate at different chlorpyrifos concentrations with a constant enzyme concentration. The enzyme was diluted with CHES buffer (pH 9.0) containing 0.1 mM CoCl₂ in the presence of bovine serum albumin at a concentration of 1 mg/ml, which was used to stabilize the activity. The change in absorbance was measured with a Beckman model DU-60 spectrophotometer for 5 min at 37°C. All assays were performed in triplicate. The kinetic constants (K_m and k_{cat}) were obtained by fitting the data to a Lineweaver-Burk reciprocal plot and a Hanes-Woolf plot.

To determine the specific activities toward other substrates, purified enzymes were resuspended in 1 ml of 50 mM phosphate-citrate buffer (pH 8.0) containing 0.1 mM CoCl₂ and either 0.15 mM paraoxon, 0.15 mM parathion, 0.25 mM methyl parathion, 0.1 mM coumaphos, or 0.2 mM chlorpyrifos in 10% methanol at 37°C for 2 min to measure the hydrolysis rate. The rates of methyl parathion, paraoxon, and parathion hydrolysis were measured by monitoring the appearance of *p*-nitrophenolate ions at 412 nm ($\epsilon_{412} = 16,500 \text{ M}^{-1} \text{ cm}^{-1}$) (14, 15). The rate of coumaphos hydrolysis was determined by measuring the production of chlorferon at 348 nm ($\epsilon_{348} = 9,100$ at pH 8.0) (6).

RESULTS AND DISCUSSION

DNA shuffling and screening for improved OPH activity against chlorpyrifos. Chlorpyrifos, a commonly used insecticide with a reported half-life of 10 to 120 days in soil (16), was chosen to demonstrate the utility of directed evolution to improve the hydrolysis rate of OPH toward a very poorly hydrolyzed substrate. Since chlorpyrifos is not readily accessible across the cell membrane, OPH was targeted directly to the

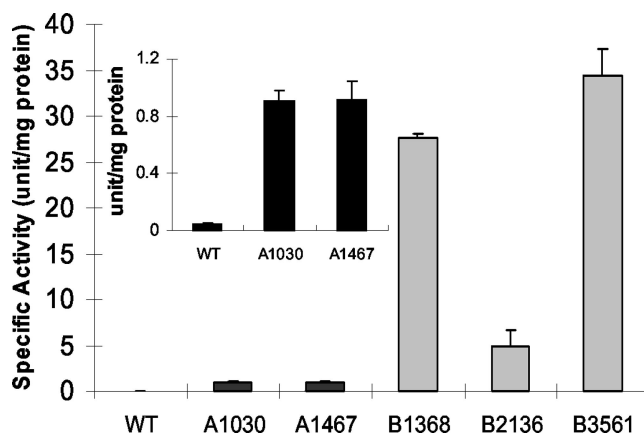


FIG. 1. Chlorpyrifos hydrolysis by purified OPH variants. Specific activities of first-round (black bars) and second-round (gray bars) variants are compared.

cell surface using an INP anchor as described previously (14). To increase the sequence diversity, the best variant, 22A11 (A14T, A80V, K185R, H257Y, I274N), obtained from improved degradation of methyl parathion (3), was used as a second template in addition to the wild-type *opd* gene. The two templates were subjected to DNA shuffling as described previously (17).

Unlike methyl parathion, the hydrolyzed products of chlorpyrifos do not have a visible color, and a modified plate method was developed for rapid prescreening. The rationale for this modified procedure is based on the highly insoluble nature of chlorpyrifos (~1.12 mg/liter) and the formation of clear haloes as a result of hydrolysis. Potential improved variants were identified based on the formation of a clear halo that is at least three times larger than that formed by cells expressing wild-type OPH. Approximately 4,700 colonies were screened in the first round, and only two clones (A1030 and A1467) were identified. Such a low frequency of improvement was surprising and may reflect the fact that chlorpyrifos is a very inefficient substrate for OPH. Perhaps accumulation of multiple additive mutations is needed to achieve significantly improved hydrolysis. To achieve higher levels of substitution, the first-round variants were used as templates for the second round of DNA shuffling and screening. Of the 7,100 colonies screened, only three clones were selected for formation of haloes larger than that of the best first-round variant, A1467.

The amount of INP-OPH expressed from the selected variants was probed by Western blot analysis with INPNC antisera (data not shown). Essentially the same expression relative to that of the wild-type OPH was observed, indicating that changes in the activity were not the result of changes in protein expression.

Characterization of improved variants. All variants identified by the plate assay were excised from the INP anchor, and a His tag was added to the N terminus to allow easy purification of the variants. Consistent with the plate assay results, all variants exhibited significantly higher hydrolytic activities for chlorpyrifos (Fig. 1), which was virtually not attacked by the wild-type OPH. Although the frequency of improved hydrolysis of chlorpyrifos was low, the best first-round variant, A1467, hydrolyzed chlorpyrifos 19 times faster than the wild-type en-

TABLE 1. Kinetic parameters for the hydrolysis of chlorpyrifos by the variants

OPH variant	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} s^{-1}$)
Wild type	0.26	73.8	2.8×10^5
A1030	0.51	2,610	5.1×10^6
A1467	0.27	1,430	5.3×10^6
B1368	0.22	21,500	9.8×10^7
B2136	0.032	3,540	1.1×10^7
B3561	0.14	30,900	2.2×10^8
22A11	0.19	20,100	1.0×10^8

zyme. An additional round of screening resulted in variants with hydrolytic rates enhanced further by at least 100-fold. The best variant, B3561, exhibited a 715-fold increase in specific activity. This superior variant hydrolyzed chlorpyrifos at a rate similar to the wild-type OPH rate of hydrolysis of paraoxon.

To further study the progress of the evolutionary process, the catalytic constants k_{cat} and K_m were measured for all variants, and the results are summarized in Table 1. Although the values for the wild-type OPH were somewhat different than those reported previously (6), the measured k_{cat} of $73.8 s^{-1}$ and K_m of 0.26 mM were consistent with the poorly hydrolyzable nature of chlorpyrifos. First-round variants showed k_{cat} values increased up to 35-fold but also a modest increase in K_m values. The overall improvement in the catalytic efficiencies (k_{cat}/K_m) was in line with the improvements in hydrolytic rate. The effect on k_{cat} values was more prominent because of the relatively high concentration (0.75 mM) of chlorpyrifos used in our screening assay, which favors the selection of mainly variants with improved k_{cat} values. Almost all second-round variants had significantly increased k_{cat} values (up to 420-fold increase for B3561) and a modest decrease in K_m . The only exception is B2136, which showed a substantial increase in binding affinity with an 8-fold reduction in K_m but only a 12-fold increase in k_{cat} . These improvements are the most useful for the detoxification and detection of chlorpyrifos at lower concentrations. The best variant (B3561) displayed a 725-fold increase in catalytic efficiency, and the measured k_{cat}/K_m value of $2.2 \times 10^8 M^{-1} s^{-1}$ is similar to the reported value for hydrolysis of paraoxon by the wild-type OPH (7).

TABLE 2. Amino acid substitutions of the variants

Variant	Amino acid substitutions
First-round shuffling variants	
A1030	A14T, A80V, K185R, I274N
A1467	A14T, R67H, A80V, L87S, Q148R, K185R
Second-round shuffling variants	
B1368	A14T, A80V, K185R
B2136	A14T, A80V, K185R, H254R, I274N
B3561	A14T, L17P, A80V, V116I, K185R, A203T, I274N, P342S

To investigate whether the increase in hydrolytic rate for chlorpyrifos resulted in changes in substrate preference, the ability of B3561 to hydrolyze substrates containing different leaving groups and either ethyl or methyl as the two nonleaving group substituents was tested (Fig. 2). Contrary to other reports (2, 4), extending the substrate preference to include chlorpyrifos had no adverse effects on the hydrolytic rates for all other substrates tested. Rather, the hydrolytic rates were universally improved from 12- to 39-fold. Considering that wild-type OPH hydrolyzes paraoxon close to the diffusion-controlled limit (7), the 39-fold improvement in hydrolysis by B3561 makes this variant one of the most efficient enzymes available to attack a wide spectrum of organophosphorus nerve agents.

Sequence and structural analysis of variants. All variants were sequenced, and their amino acid substitutions are listed in Table 2. Although the A14T mutation was found in all variants, this substitution is located in the signal sequence of OPH. No noticeable difference in activity was observed when this substitution was removed, suggesting that it is unlikely to contribute to improved activity. The presence of the A80V and K185R mutations appears to be the most beneficial, as the B1368 variant carrying only these two mutations exhibited a 290-fold increase in k_{cat} , while an additional mutation, I274N, in A1030 reduced k_{cat} by 8-fold. Similarly, k_{cat} decreased by 15-fold when the extra mutations R67H, Q148R, and L87S

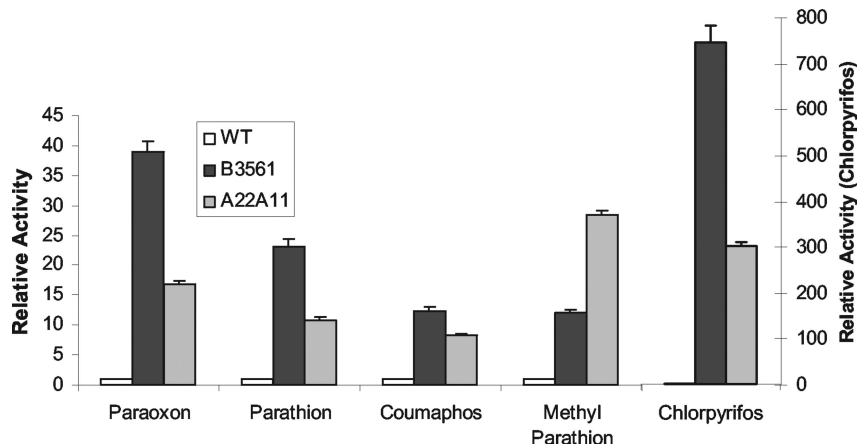


FIG. 2. Hydrolysis of paraoxon, parathion, methyl parathion, coumaphos, and chlorpyrifos. Relative activities of purified wild-type (WT) OPH, B3561, and 22A11 are compared.

were present in A1467. This result indicates that AV80 and K185R may help increase the overall hydrolysis rate through elevation of k_{cat} , whereas the other substitutions have an opposite effect. The K185R mutation is situated in a remote external helix near the leaving-group binding pocket. It has been suggested that the conversion of K185R allows the formation of hydrogen bonds to Glu219 or Glu181, resulting in interactions that stabilize the overall structure (20). This structural change may stabilize OPH in a more active conformation, thereby increasing not only the hydrolytic efficiency for chlorpyrifos but also the global hydrolysis rate of all other substrates tested. The A80V mutation, on the other hand, lies near the surface of OPH, and its effects on improving activity are unclear. However, these two substitutions appear to act in a synergistic fashion, as a mutant containing only the K185R substitution is completely inactive (unpublished results).

Although most substitutions have only a modest impact on substrate binding, the presence of the H254R substitution in B2136 reduced K_m by eightfold. The same mutation in wild-type OPH has also been shown to lower K_m by 8-fold for paraoxon and by 11-fold for diisopropylfluorophosphonate (4). This substitution is directly located in the large binding pocket and has been reported to result in a loss of one metal ion from each binding site (13). This permits more flexibility for substrate binding, leading to the increase in binding affinity (11).

It is interesting that some of the important substitutions (A80V, I274N, and K185R) presented in B3561 are already found in the parental template, 22A11. Not surprisingly, chlorpyrifos degradation was also improved for 22A11, although the hydrolysis rate is only 38% that of B3561. A comparison of the specific activities toward other substrates revealed that B3561 also hydrolyzed all substrates tested faster, except for methyl parathion (Fig. 2). These results suggest that these three mutations (A80V, I274N, and K185R) may be important in increasing the global hydrolysis rate for both variants. The further enhancement in hydrolysis of chlorpyrifos as well as other substrates by B3561 is clearly the contribution of the additional V116I and A203T substitutions. The A203T mutation, in particular, is located on the loop adjacent to the His201 and His230 residues, which are involved in metal-ion coordination (20). The Thr substitution may allow a potential ionic bridge formation with an adjacent Gln206 to provide a wider opening to the active site. Since chlorpyrifos contains 3,5,6-trichloro-2-pyridinol as the leaving group, which is bulky compared to *p*-nitrophenol in paraoxon, the A203T mutation may further improve the accessibility of chlorpyrifos as reflected in both increased k_{cat} and decreased K_m values compared to 22A11 (Table 1).

Conclusion. In this study, we have demonstrated the utility of directed evolution to create OPH variants with enhanced catalytic efficiency toward a poorly hydrolyzable substrate, chlorpyrifos. One novel variant (B3561) was obtained after two rounds of DNA shuffling and screening and demonstrated 725-fold improvement in chlorpyrifos hydrolysis. The resulting k_{cat}/K_m value of $2.2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ is close to the reported diffusion-controlled limit for enzymatic catalysis. The use of this novel variant could significantly improve the efficiency of chlorpyrifos decontamination.

In addition to chlorpyrifos, the best variant, B3561, also degrades several other pesticides 12- to 39-fold faster than the

wild-type OPH, suggesting that some of these substitutions may be of great importance in enhancing the global hydrolytic efficiency of OPH. Unlike other approaches (4, 8), which focus on engineering the active-site residues, our approach surveys the entire protein sequence and provides important substitutions that are far away from the active sites. Considering that only a few substitutions are responsible for this improvement in hydrolysis, directed evolution represents a powerful and simple method to create a cocktail of novel biocatalysts with dramatically improved rates of hydrolysis of other very poorly hydrolyzed targets, such as demeton-S, sarin, and soman.

ACKNOWLEDGMENTS

This work was supported by a grant from the USDA (99-35102-5897).

We thank Mark Shimazu for constructing the plasmid pINCOP.

REFERENCES

1. Bushway, R. J., and Z. Fan. 1998. Complementation of GC-AED and ELISA for the determination of diazinon and chlorpyrifos in fruits and vegetables. *J. Food Prot.* **61**:708–712.
2. Chen-Goodspeed, M., M. A. Sogorb, F. Wu, S.-B. Hong, and F. M. Raushel. 2001. Structure determinants of the substrate and stereochemical specificity of phosphotriesterase. *Biochemistry* **40**:1325–1331.
3. Cho, C. M.-H., A. Mulchandani, and W. Chen. 2002. Bacterial cell surface display of organophosphorus hydrolase for selective screening of improved hydrolysis of organophosphate nerve agents. *Appl. Environ. Microbiol.* **68**:2026–2030.
4. Di Sioudi, B. D., C. E. Miller, K. Lai, J. K. Grimsley, and J. R. Wild. 1999. Rational design of organophosphorus hydrolase for altered substrate specificities. *Chem.-Biol. Interact.* **119–120**:211–223.
5. Donarski, W. J., D. P. Dumas, D. P. Heitmeier, V. E. Lewis, and F. M. Raushel. 1989. Structure-activity relationships in the hydrolysis of substrates by the phosphotriesterase from *Pseudomonas diminuta*. *Biochemistry* **28**:4650–4655.
6. Dumas, D. P., S. R. Caldwell, J. R. Wild, and R. M. Raushel. 1989. Purification and properties of the phosphotriesterase from *Pseudomonas diminuta*. *J. Biol. Chem.* **261**:19659–19665.
7. Griffiths, A. D., and D. S. Tawfik. 2003. Directed evolution of an extremely fast phosphotriesterase by *in vitro* compartmentalization. *EMBO J.* **22**:24–35.
8. Hill, C. M., W.-S. Li, J. B. Thoden, H. M. Holden, and F. M. Raushel. 2003. Enhanced degradation of chemical warfare agents through molecular engineering of the phosphotriesterase active site. *J. Am. Chem. Soc.* **125**:8990–8991.
9. Hong, S. B., and F. M. Raushel. 1999. Stereochemical constraints on the substrate specificity of phosphotriesterase. *Biochemistry* **38**:1159–1165.
10. Inoue, H., H. Nojima, and H. Okayama. 1990. High efficiency transformation of *Escherichia coli* with plasmids. *Gene* **96**:23–28.
11. Koča, J., C.-G. Zhan, R. C. Rittenhouse, and R. L. Ornstein. 2001. Mobility of the active site bound paraoxon and sarin in zinc-phosphotriesterase by molecular dynamics simulation and quantum chemical calculation. *J. Am. Chem. Soc.* **123**:817–826.
12. Lai, K. H., J. K. Grimsley, B. D. Kuhlmann, L. Scapozza, S. P. Harvey, J. J. DeFrank, J. E. Kolakowski, and J. R. Wild. 1996. Rational enzyme design: computer modeling and site-directed mutagenesis for the modification of catalytic specificity in organophosphorus hydrolase. *Chimia* **50**:430–431.
13. Lai, K., K. I. Dave, and J. R. Wild. 1994. Bimetallic binding motifs in organophosphorus hydrolase are important for catalysis and structural organization. *J. Biol. Chem.* **269**:16579–16584.
14. Shimazu, M., A. Mulchandani, and W. Chen. 2001. Cell surface display of organophosphorus hydrolase using ice nucleation protein. *Biotechnol. Prog.* **17**:76–80.
15. Shimazu, M., A. Mulchandani, and W. Chen. 2001. Simultaneous degradation of organophosphorus pesticides and *p*-nitrophenol by a genetically engineered *Moraxella* sp. with surface-expressed organophosphorus hydrolase. *Biotechnol. Bioeng.* **76**:318–324.
16. Singh, B. K., A. Walker, J. A. W. Morgan, and D. J. Wright. 2003. Effects of soil pH on the biodegradation of chlorpyrifos and isolation of a chlorpyrifos-degrading bacterium. *Appl. Environ. Microbiol.* **69**:5198–5206.
17. Stemmer, W. P. C. 1994. DNA shuffling by random fragmentation and

- reassembly: in vitro recombination for molecular evolution. *Proc. Natl. Acad. Sci. USA* **91**:10747–10751.
18. **Watkins, L. M., J. M. Kuo, M. Chen-Goodspeed, and F. M. Raushel.** 1997. A combinatorial library for the binuclear metal center of bacterial phosphotriesterase. *Proteins Struct. Funct. Genet.* **29**:553–561.
19. **Wu, F., W.-S. Li, M. Chen-Goodspeed, M. A. Sogorb, and F. M. Raushel.** 2000. Rationally engineered mutants of phosphotriesterase for preparative scale isolation of chiral organophosphates. *J. Am. Chem. Soc.* **122**:10206–10207.
20. **Yang, H., P. D. Carr, S. Y. McLoughlin, J. W. Liu, I. Horne, X. Qiu, C. M. J. Jeffries, R. J. Russell, J. G. Oakeshott, and D. L. Ollis.** 2003. Evolution of an organophosphate-degrading enzyme: a comparison of natural and directed evolution. *Protein Eng.* **6**:135–145.