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Factors Influencing Parathion Degradation by Recombinant *Escherichia coli* with Surface-Expressed Organophosphorus Hydrolase

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A unique approach for organophosphorus pesticides detoxification was developed previously by anchoring organophosphorus hydrolase (OPH) onto the surface of *Escherichia coli* with a tightly regulated *tac* promoter. The resulting recombinant cells degraded parathion very effectively without the diffusional limitation observed in cells expressing OPH intracellularly. However, the precise conditions for surface targeting or pesticide degradation were not fully understood. In this paper, several factors influencing parathion degradation were investigated. Production of active OPH onto the cell surface was highly host-specific; a high rate of parathion degradation was observed from strains JM105 and XL1-Blue, which regulated production of the OPH fusion very tightly. However, in the absence of ampicillin selection, plasmids were only favorably maintained in strain XL1-Blue. OPH activity was highly dependent on growth conditions. Optimal OPH activity was observed when cells were grown in Luria–Bertani (LB)-buffered medium at 37 °C. OPH activity was further improved by supplementing the growth medium with cobalt chloride, which favors the formation of the metal active center. The timing of cobalt addition also influenced parathion degradation. Maximum OPH activity was obtained by adding cobalt to induced cultures during the late stationary phase. The resulting cultures grown under the optimized conditions had an eight-fold increase in parathion degradation.

Introduction

Organophosphates are a group of highly toxic compounds that are used extensively as agricultural and domestic pesticides. Organophosphorus hydrolase (OPH) has been isolated from soil microorganisms (*1, 2*) and shown to be effective in degrading a range of organophosphate esters (*3*). Since the economics of culturing the native soil bacteria in bioreactors is not very attractive, because of slow specific growth rates, the *opd* gene has been cloned into *Escherichia coli* (*4*), insect cell (fall armyworm) (*5*), *Streptomyces* (*6*), and soil fungus (*7, 8*). Recombinant OPHs have been demonstrated to be equally effective in hydrolyzing various organophosphorus pesticides and nerve agents.

Recently, active OPH was successfully anchored and displayed onto the cell surface of *Escherichia coli* using an Lpp-OmpA(46–159) fusion system (*9*). Although recombinant strains expressing OPH constitutively onto the cell surface degraded parathion very effectively, they were relatively unstable. A gradual decline in OPH activity was observed over a period of 3 months. It was reasoned that constitutive expression of a surface protein leads to high structural instability of the expression vector. To address this problem, a new plasmid was constructed to express OPH onto the cell surface under control of a tightly regulated *tac* promoter system. The resulting plasmid was very stable, and *E. coli* cells transformed with it degraded parathion and paraoxon very effectively without the diffusional limitation observed in cells expressing OPH intracellularly. Cultures with surface-expressed OPH also exhibited a very long shelf life, retaining 100% activity over a period of 1 month. It is anticipated that immobilization of these live biocatalysts onto solid supports will provide an attractive and economical means for pesticide detoxification in place of immobilized enzymes or immobilized whole cell expressing OPH intracellularly. However, the precise conditions for surface targeting or pesticide degradation are not fully understood.

To gain a better understanding of this new system and to explore its potential for pesticide detoxification, we investigated several factors influencing OPH expression and parathion degradation. The effects of strain selection, growth conditions, cofactor addition, and isopropyl-β-D-1-thiogalactopyranoside (IPTG) induction were investigated to define the optimal conditions for parathion degradation. In addition, plasmid stability of the resulting recombinant cells was studied.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions. *E. coli* strains JM105 (*end*A1, *thi*, *rps*L, *sbc*B15, *hsd*R4, Δ(*lac-pro*AB), [F′, *tra*D36, *pro*AB, *lac*I^{q}ZDM15]), XL1-Blue (*rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_k^-, m_k^+), *sup*E44, *rel*A1, *lac* [F′, *pro*AB, *lac*I^{q}ZDM15, Tn10 (Tet^r)]), DH5α F′ (F′ *ϕ80dlac*Δ*M*15 Δ(*lacZYA-argF*)*U169 deoR*

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recA1 endA1 hsdR17 (r_k^-, m_k^+) *supE44*λ^- *thi-1 gyr*A96 *relA1*), and HMY101 (lac *rmf*$^-$) were used in this study.

Plasmid pOPK132 was used for expressing Lpp-OmpA-OPH on the cell surface (*9*). Expression of OPH is tightly regulated by a *tac* promoter because of the presence of the *lacI*^{q} gene on the plasmid. Plasmid pWM513, which contains the *opd* gene from *Flavobacterium sp.*, was used as a control for the production of native OPH in the cytoplasm (*10*).

Strains bearing plasmid were grown in Luria−Bertani (LB) media supplemented with ampicillin to a final concentration of 100 μg/mL. Induction was achieved by adding 0.5 mM IPTG to the cultures at an OD_{600} of 0.5, unless otherwise specified. In most experiments, the medium was also buffered to pH 7.0 with 0.017 M KH_2PO_4 and 0.072 M K_2HPO_4, and supplemented with 1 mM $CoCl_2$. Cells were grown in 250-mL flasks in a Innova 4000 shaker (New Brunswick Scientific) with vigorous agitation (300 rpm) at 22, 30, or 37 °C.

Cell Fractionation. Cultures were grown at 37 °C and induced with 0.5 mM IPTG. After 48 h, cells were harvested by centrifugation at 5000*g* for 10 min, washed once with 25 mM Tris-HCl pH 7.5, and suspended in 10 mL of the same buffer containing 1 mM EDTA and 100 mg/mL lysosyme at 4 °C. After 3−5 min of incubation, cells were disrupted by a French pressure cell at 9000−11 000 psi. The cellular debris were removed by centrifugation at 10000*g* for 15 min. The total membrane fraction was recovered by centrifugation at 115000*g* for 1 h at 4 °C.

Organophosphorus Hydrolase Assay. Samples were suspended in 15% (w/v) sucrose, 15 mM Tris-HCl (pH 7.8). Assays were conducted in 1.5-mL disposable methacrylate cuvettes (Fisher). For each assay, 900 μL of samples (~1 OD_{600} cells) was combined with 100 μL of 6.5 mM parathion (Supleco) in 10% methanol. Reaction mixtures were incubated at room temperature or 30 °C (as indicated) during which time the changes in absorbance (410 nm) were monitored. Activities are expressed as micromoles of parathion hydrolyzed per minute, per OD_{600} whole cells (ϵ_{410} = 16 500 M^{-1} cm^{-1} for *p*-nitrophenol).

Plasmid Stability Studies. Bacterial cells bearing pOPK132 were grown in LB medium buffered with 0.017 M KH_2PO_4 and 0.072 M K_2HPO_4 without ampicillin. Once the cultures reached an OD_{600} of 0.5, 0.5 mM IPTG was added. Samples were taken immediately as well as 6, 22, and 48 h post-induction. After appropriate dilutions were made, 100 μL of each sample was plated on LB/agar plates with and without ampicillin (100 μg/mL). Bacterial colonies were counted after 16 h of incubation at 37 °C. Plasmid stability was expressed as the ratio between the number of colonies formed on plates with and without ampicillin.

Results

Effect of Host Strains on OPH Activity. To identify a superior host for anchoring OPH onto the surface and to maximize parathion degradation, four different cell lines were examined based on (i) OPH activity; (ii) percentage of OPH on the surface; and (iii) plasmid stability. Three commonly used *E. coli* strains, JM105, XL1-Blue, and DH5αF′, were chosen to investigate the effect of *lacI*^{q} background on OPH expression and activity. *E. coli* strain HMY101, which cannot form inactive ribosome dimers (*11*) during stationary growth, was chosen for the investigation of the effect of stationary phase response on OPH expression. The resulting OPH activities from these recombinant strains carrying plasmid pOPK132 after 1 and 2 days are shown in Figure 1. Consistent with previous observations with strain JM105, OPH activity was significantly higher in the second-day cultures. OPH activity was noticeably lower in strain DH5αF′ in both the first- and second-day cultures. It is possible that basal expression of the OPH fusion was higher in this non-*lacI*^{q} strain, leading to increased plasmid instability and lower observed OPH activity (*9, 12*).

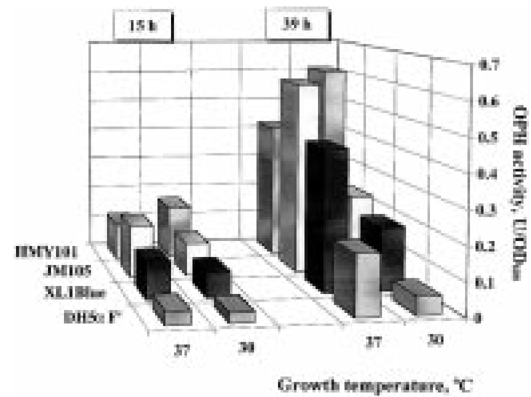

Figure 1. OPH activity of strains DH5αF′, XL1-Blue, JM105, and HMY101 carrying plasmid pOPK132 at 30 and 37 °C. Cells were grown in LB medium, and samples were taken 15 and 39 h after induction.

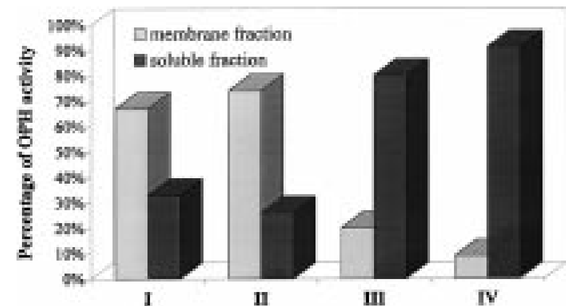

Figure 2. Distribution of OPH activity between soluble and membrane fractions. Cultures were grown at 37 °C and induced with 0.5 mM IPTG. Cell lysates were fractionated as described in the Materials and Methods. I: XL1-Blue:pOPK132; II: JM105:pOPK132; III: HMY101:pOPK132; IV: JM105:pWM513.

To confirm that the resulting OPHs were displayed on the surface, cultures were harvested in late exponential phase, lysed in a French pressure cell, and separated into soluble and membrane fractions by high-speed centrifugation. The resulting distribution of OPH activity between the soluble and membrane fractions is shown in Figure 2. As expected, >90% of the OPH activity was found in the soluble fraction of JM105:pWM513, which expresses OPH intracellularly. In contrast, cultures carrying pOPK132 had >70% of the OPH activity associated with the membrane fractions, except for HMY101. Even though cultures of HMY101 had high OPH activity, this host strain is not desirable because the majority of the activity was intracellular.

Effect of Growth Medium, Growth Temperature, and Induction on OPH Activity. The effect of pH variation was investigated by culturing the recombinant cells in LB and LB-buffered medium. Cell growth rates were essentially the same between the two media. However, OPH activity was 30 and 70% higher in LB-buffered medium for strain XL1-Blue and JM105, respectively (Figure 3).

Temperature is a key factor in determining protein processing (*13, 14*). For β-lactamase, only 20% of the

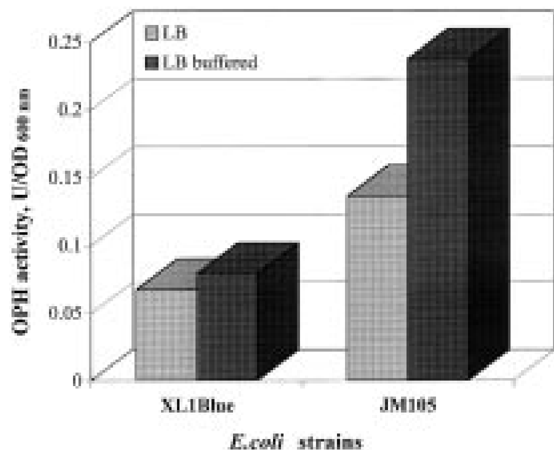

Figure 3. OPH activity from JM105:pOPK132 and XL1-Blue: pOPK132 cells grown in LB and LB-buffered media. Samples were taken 15 h after induction.

enzymes were surface exposed at 37 °C compared with ~100% at 24 °C (*15*). Cultures expressing OPH constitutively on the surface did not survive at 37 °C and can only be grown at 30 °C or lower temperatures. To determine how growth temperature could influence surface expression of OPH from an inducible system, three different growth temperatures (37, 30, and 22 °C) were investigated. As depicted in Figure 4, cultures remained viable at all three temperatures, and parathion degradation was observed. In contrast to previous studies with constitutive expression (*9*), our results indicate that OPH activity was the highest in both strains at 37 °C, a temperature that is more favorable for growth.

The effect of induction on OPH activity was also investigated. Our goal was to define the minimum IPTG needed to achieve full induction. As evident from Figure 4, a considerable level of OPH activity was obtained with as low as 0.2 mM IPTG induction at 37 °C. At higher IPTG concentrations, OPH activity was increased by almost twofold for strain XL1-Blue, whereas a small decline in OPH activity was observed for strain JM105.

Stability of Recombinant Strains Carrying Plasmid pOPK132. Detoxification of pesticides using recombinant cells in a large-scale immobilized bioreactor requires the production of stable and active cultures without the supply of antibiotics. Culture stability is even more important if the regeneration of live biocatalysts in the immobilized bioreactor is desirable. Almost 100% of JM105 and XL1-Blue cultures grown in the absence of ampicillin contained plasmid before induction (Table 1). After IPTG addition, a decline in the number of plasmid-bearing cells was observed in both cultures. For the XL1-Blue strain, cultures were able to withstand this initial shock, and ~70% of the cells still contained plasmid after 48 h. In contrast, no plasmid-bearing cells remained for cultures of JM105 after 48 h. From an economic standpoint, cultures of XL1-Blue are superior for industrial applications because stable and active cultures can be maintained in the absence of selection.

Effect of Cobalt Ion on OPH Activity. Catalytically active OPH requires the presence of zinc or cobalt as a cofactor. If zinc/cobalt is removed, enzyme activity will be lost (*16*). At present, it is still unclear how cobalt addition affects the overall expression and activity of OPH on the cell surface, so experiments were conducted to investigate this relationship. The presence of 1 mM $CoCl_2$ in the growth medium increased OPH activity by almost twofold (Figure 5 column III and IV), an observation consistent with that of Serdar et al. (*4*). However, growth rate and final cell density were markedly reduced in cobalt supplemented medium, presumably due to the toxic effect of cobalt. Because cobalt is needed only as a metal center for OPH, we reason that it may be possible to achieve high OPH activity even if cobalt is added only during cultivation. This possibility was explored by varying the timing of $CoCl_2$ addition (Figure 5). Addition of cobalt in conjunction with induction resulted in almost the same OPH activity as addition of cobalt prior to inoculation. Cell growth was also greatly reduced, as in the former case. In contrast, addition of cobalt to stationary-phase cultures (17 h after induction in our experiment) resulted not only in an additional twofold increase in OPH activity, but normal cell growth was also restored. The resulting parathion degradation from strain XL1Blue:pOPK132, grown under these optimal conditions, was eightfold higher compared with strain JM105:pOP131, expressing OPH constitutively on the surface (*9*).

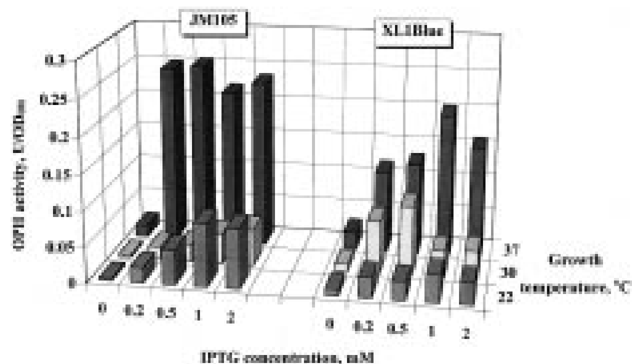

Figure 4. Effect of temperature and induction on OPH activity from JM105:pOPK132 and XL1-Blue:pOPK132 cells grown in LB-buffered medium at 37 °C. Samples were taken 15 h after induction.

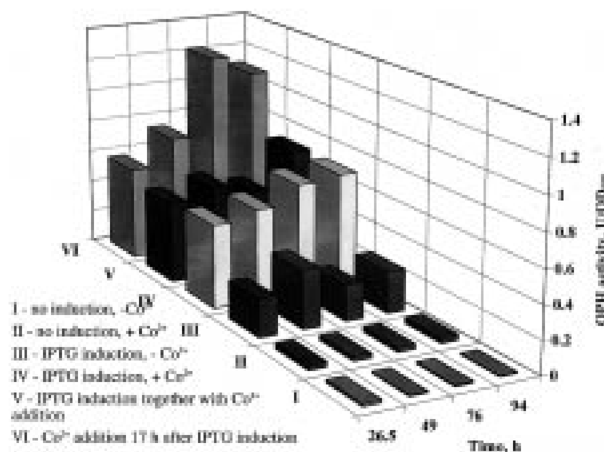

Figure 5. Effect of cobalt addition on OPH activity. Cultures of XL1-Blue:pOPK132 were grown in LB-buffered medium at 37 °C, and samples were taken at various time points.

Table 1. Plasmid Stability of Strains JM105 and XL1-Blue Carrying pOPK132 in the Absence of Ampicillin

	percentage of plasmid-bearing cells	
post-induction, h	XL1-Blue	JM105
0	100	100
6	95	63
22	70	0
48	67	0

Discussion

OPH activity from DH5αF′ was consistently lower than that from both JM105 and XL-Blue. This result indicates that the level of basal expression may have an adverse effect on expressing OPH on the surface, as high-level induction was only possible under very tightly regulated conditions. This result is in line with previous observations, indicating that constitutive expression of the OPH fusion on the cell surface is detrimental (*9*). Because the majority of OPH activity was accumulated during the stationary phase and OPH synthesis is unlikely to occur under starvation condition, stationary-phase translocation or processing of previously synthesized OPH fusions may be a plausible explanation. The possible link of stationary phase response with surface expression was demonstrated by introducing plasmid pOPK132 into the *E. coli* strain HMY101, which is deficient in forming inactive ribosome dimers during stationary growth. Even though OPH activity was high in strain HMY101, most of the activity was intracellular, which is indicative of improper processing.

Growth conditions affect OPH expression greatly; OPH activity was enhanced when pH variation during cultivation was reduced in buffered-LB medium. Furthermore, a more growth-favorable temperature also appears to enhance OPH expression and translocation. When expression of OPH fusions was induced only during exponential growth, higher OPH activity can be achieved at 37 °C. This result is in sharp contrast with the lethal effect of high-level, constitutive OPH expression onto the surface at the same temperature (*9, 15*). Similar adverse effects were illustrated by the IPTG induction experiments. OPH activity reached a maximum at medium induction and then declined slightly. High-level induction and hence translocation of OPH again appears to have a negative impact on cell growth and OPH expression. A similar observation on surface expression of β-lactamase has also been reported by others (*17*). Induction also had an effect on plasmid stability as the number of plasmid-bearing cells sharply declined upon induction in the absence of selection, probably due to the metabolic burden of excessive OPH expression on the surface.

Serdar et al. (*4*) reported that the presence of a metal center is essential for achieving high OPH activity by stabilizing the enzymes. To expedite the formation of the cobalt centers, cultures of XL1Blue:pOPK132 were grown in the presence of $CoCl_2$. Even though OPH activity was enhanced, the presence of $CoCl_2$ was inhibitory to cell growth. Because the OPH domain is anchored on the cell surface, we argue that cobalt uptake may not be necessary, and they might be readily available to the histidine cluster of surface-exposed OPHs. This situation was demonstrated by adding cobalt to culture that had already reached late stationary phase. The resulting OPH activity was twofold higher and cell growth was no longer inhibited. This result suggests that it may even be possible to first harvest the cells and then incubate the cells with cobalt. Such a behavior was indeed observed. A gradual increase in parathion degradation was detected with increasing incubation time (data not shown), but this strategy offers no obvious benefit because prolonged incubation is still necessary for full activation.

Acknowledgment

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