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Export of Methyl Parathion Hydrolase to the Periplasm by the Twin-Arginine Translocation Pathway in *Escherichia coli*

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The uptake of organophosphates (OPs) is a rate-limiting factor in whole-cell biocatalysis systems. Here, we report the periplasmic secretion of methyl parathion hydrolase (MPH) by employing the twin-arginine translocation (Tat) pathway in *Escherichia coli*. The twin-arginine signal peptide of trimethylamine *N*-oxide reductase (TorA) from *E. coli* was used for exporting MPH to the periplasm of *E. coli*, alleviating the substrate uptake limitation. A periplasmic expression vector, pUTM18, coding for TorA−MPH was constructed, and the periplasmic secretion and functionality of MPH were demonstrated by cell fractionation, immunoblotting, and enzyme activity assays. The strain expressing periplasmic MPH showed 3-fold higher whole-cell activity than the control strain expressing cytoplasmic MPH. Suspended cultures also exhibited good stability, retaining almost 100% activity over a period of 2 weeks. Owing to their high activity and superior stability, these "live biocatalysts" are ideal for large-scale detoxification of OPs.

KEYWORDS: Tat pathway; twin-arginine signal peptide; periplasmic secretion; methyl parathion hydrolase

INTRODUCTION

Synthetic organophosphates (OPs) are a group of highly toxic chemicals widely used to control various agriculture pests, accounting for ∼38% of total pesticides used globally (*1*). Over 40 million kilograms of OP pesticides are used annually in the U.S., with another 20 million kilograms produced for export (*2*). OP pesticides are acetylcholinesterase (AChE) inhibitors, and various clinical effects can occur from OP poisoning in humans (*3*). The growing public concern about their safety and the widespread use of OPs in modern agriculture has stimulated the development of effective and safe remediation strategies for detoxification of OPs.

Organophosphorus hydrolase (OPH), encoded by the *opd* gene of *Pseudomonas diminuta* MG and *Flavobacterium* sp. strain ATCC27551, is a homodimeric phosphotriesterase that can hydrolyze a wide range of OPs (*4*, *5*). Hydrolysis of OPs by OPH reduces their toxicity by several orders of magnitude (*3*). Practical applications of large-scale enzymatic degradation have always been limited by the cost of purification and stability of OPH. Although the use of whole cell as biocatalysts is an alternative strategy for treatment of OPs, the inaccessibility of the pesticides across the cell membrane reduces the overall catalytic efficiency (*2*, *6*). This barrier of substrate transport can be overcome by displaying OPH on the cell surface. However, surface expression of OPH resulted in instability of the cell membrane and growth inhibition of the cells, arising from increased metabolic burden placed on the cell (*2*, *6*).

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Periplasmic secretion of target proteins can be an alternative strategy for reducing the substrate diffusion barrier in whole-cell biocatalysis systems (*7*). The twin-arginine translocation (Tat) system is a bacterial protein export pathway with the remarkable ability to transport folded proteins across the cytoplasmic membrane (*8*−*10*). Proteins are targeted to a membrane-embedded Tat translocase by specialized N-terminal twin-arginine signal peptides bearing a consensus motif of SRRxFLK. The nearly invariant twin-arginine dipeptide is essential for efficient Tat targeting (*9*, *11*, *12*). Tat signal peptides consist of three domains: a positively charged N-terminal domain (n-region), a hydrophobic domain (h-region), and a C-terminal domain (c-region) (*11*, *13*). In *E. coli*, the Tat translocase consists of the TatA, TatB, and TatC proteins. These three classes of membrane proteins form two types of high-molecular-weight complexes, including the TatBC signal recognition complex and TatA transport channel complex (*14*−*16*). The TatBC complex binds a Tat substrate by the specific recognition for the twin-arginine motif. The TatA complex forms a channel through which substrates are translocated across the cytoplasmic membrane.

Recently, an OP degradation gene (*mpd*) encoding methyl parathion hydrolase (MPH) was isolated from a methyl parathion-degrading bacterium *Plesiomonas* sp. strain M6, but it showed only 12% identity to *opd* gene at the amino acid level (*17*), suggesting significant novelty of the gene−enzyme system. More recently, we cloned the *mpd* gene (GenBank accession no. DQ677027) from a chlorpyrifos-degrading bacterium *Stenotrophomonas* sp. strain YC-1 (*18*). In the present study, to enable secretion of MPH to the periplasm of *E. coli*, we used the twin-arginine signal peptide of trimethylamine *N*-oxide reductase (TorA) from *E. coli* (*19*).

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Table 1. Strains, Plasmids and Primers Used in This Study

strain, plasmid, or primer	description[a]	source or literature
strains		
E. coli DH5α	*supE44* △*lacU169*(*φ80 lacZ*△M15) *recA1 endA1 hsdR17*(r_K^- m_K^+) *thi-1 gyrA relA1* F^- △(*lacZYA-argF*)	Tiangen
E. coli MC4100	F^- △*lacU169 araD139 rpsL150 relA1 ptsF rbs flb B5301*	*20*
B0D	like MC4100, △*tatB*	*20*
B1LK0	like MC4100, △*tatC*	*20*
plasmids		
pSCTorA-GFP	gene source of TorA signal peptide	*19*
pMDQ	source of *mpd* gene	*18*
pUC18	vector for construction of *torA*−*mpd* fusion gene, *lac* promoter, Ap^r	TaKaRa
pUTM18	pUC18 derivative, periplasmic expression vector coding for TorA−MPH fusion	this study
pUM18	pUC18 derivative, cytoplasmic expression vector coding for mature MPH	this study
primers		
P1	GAATTCCTCTAGAGGGTATTAATAATGAACAATAACGATCTCTTT	this study
P2	GGATCCCGCCGCTTGCGCCGCAGTCGC	this study
P3	ggATCCGCCGCACCGCAGGTGcgc	this study
P4	AAGCTTTCACTTGGGGTTGACGAC	this study
P5	GAATTCCGCCGCACCGCAGGTGcgc	this study

[a] The restriction sites in the primers (5′ → 3′) are underlined.

MATERIALS AND METHODS

Strains, Plasmids, Primers, and Culture Conditions. The strains, plasmids, and primers used in this study are described in **Table** 1. *E. coli* DH5α was used for constructing recombinant plasmids. *E. coli* MC4100 and its *tat* mutant strains (*20*) were used for recombinant MPH expression. *E. coli* strains bearing plasmids were grown in Luria−Bertani (LB) medium (*21*) supplemented with 100 μg/mL ampicillin. Expression of recombinant proteins was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 12 h at 30 °C when cells were grown to an OD_{600} of 0.5.

Plasmid Construction. The *torA* gene fragment encoding the signal peptide and the first four amino acid residues of the mature TorA was amplified by polymerase chain reaction (PCR) from plasmid pSCTorA-GFP using primers P1 and P2. The PCR products were digested with *Eco*RI and *Bam*HI, and then ligated into similarly digested pUC18 to generate pUT18. The *mpd* gene was amplified by PCR from plasmid pMDQ using primers P3 and P4. The PCR products were digested with *Bam*HI and *Hin*dIII, and then ligated into similarly digested pUT18 to generate pUTM18. To construct a control plasmid expressing cytoplasmic MPH, the *mpd* gene was amplified by PCR from plasmid pMDQ using primers P5 and P4. The PCR products were digested with *Eco*RI and *Hin*dIII, and then ligated into similarly digested pUC18 to generate pUM18. All plasmid constructions were verified by DNA sequence analysis. Transformation of the plasmid into *E. coli* was carried out by using the $CaCl_2$ method (*21*).

Cell Fractionation. Cells were fractionated to yield cytoplasmic and periplasmic samples by the cold osmotic shock procedure as follows (*8*, *22*). Cells expressing TorA−MPH were harvested, resuspended in 5 mL of TES buffer (30 mM Tris-HCl, pH 8.0, 1 mM EDTA, 20% sucrose) and incubated at room temperature for 10 min. Cells were harvested by centrifugation at 10,000 rpm for 10 min at 4 °C, resuspended in 2 mL of ice-cold 5 mM $MgSO_4$ and incubated on ice for 10 min to generate shocked cells. Shocked cells were collected by centrifugation as above, and the supernatant was retained as the periplasmic fraction. Shocked cells were resuspended in 2 mL of ice-cold 5 mM $MgSO_4$ and sonicated for 5 × 20 s bursts, with 20 s cooling in between, at an amplitude of 15 microns. Cell debris was removed by centrifugation at 10,000 rpm for 5 min at 4 °C. Membranes were pelleted by centrifugation at 50,000 rpm for 1 h at 4 °C, and the supernatant was retained as the cytoplasmic fraction.

Western Blot Analysis. Samples of total cell lysate and cytoplasmic and periplasmic fractions were analyzed by sodium dodecyl sulfate-polyacyrlamide gel electrophoresis (SDS−PAGE) with 12% (w/v) acrylamide (*21*). After electrophoresis, the separated proteins were electroblotted overnight at 40 V to the nitrocellulose membrane (Millipore, Billerica, MA) with a tank transfer system (Bio-Rad, Hercules, CA) containing a transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol). After blocking nonspecific binding sites with 3% bovine serum albumin (BSA) in TBST buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20), the membrane was incubated with rabbit anti-MPH serum at a 1:500 dilution in TBST buffer. Subsequently, the membrane was incubated with alkaline phosphatase-conjugated goat antirabbit IgG antibody (Promega, Madison, WI) at a 1:1000 dilution. The membrane was then stained with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.0, 100 mM NaCl) for visualizing antigen−antibody conjugates.

MPH Activity Assay. Transformed cells were harvested and resuspended (OD_{600} = 1.0) in 100 mM phosphate buffer (pH 7.4). MPH activity assay mixtures (1 mL, 3% methanol) contained 50 μg/mL methyl parathion (added from a 10 mg/mL methanol stock solution), 870 μL of 100 mM phosphate buffer (pH 7.4), and 100 μL of cells. The enzyme activity was measured using a Beckman DU800 spectrophotometer at 30 °C by monitoring the increases of linear optical density over time at 405 nm as methyl parathion was hydrolyzed to *p*-nitrophenol (ε_{405} = 17,700 M^{-1} cm^{-1}). Activities were expressed as units (1 μmol of *p*-nitrophenol formed per minute) per OD_{600} whole cells.

Stability Study of Resting Cultures. Cells harboring pUTM18 were grown in 50 mL of LB medium supplemented with 1 mM IPTG and 100 μg/mL ampicillin for 24 h, washed twice with 50 mL of 150 mM NaCl solution, resuspended in 5 mL of 100 mM phosphate buffer (pH 7.4), and incubated in a shaker at 30 °C. Over a 2-week duration, 0.1 mL of sample was removed each day. Samples were centrifuged and resuspended in 0.1 mL of 100 mM phosphate buffer (pH 7.4). MPH activity assays were conducted as described above.

Biodegradation of Organophosphorus Pesticides by Periplasmic Expression Strain. For expression of recombinant proteins, *E. coli* MC4100 cells carrying pUTM18 or pUM18 were induced with 1 mM IPTG for 12 h at 30 °C. The induced MC4100 cells were harvested, followed by washing with 100 mM phosphate buffer (pH 7.4), and resuspended (OD_{600} = 1.0) in the same buffer. Subsequently, 0.4 mM methyl parathion, fenitrothion, parathion, or chlorpyrifos was added to the cell suspensions. Samples were incubated for 2 h at 30 °C with shaking and measured for the residual pesticides by gas chromatography as described previously (*18*).

RESULTS AND DISCUSSION

Construction and Expression of a TorA−MPH Chimera. The Tat pathway has been widely used for the export of heterologous proteins, such as green fluorescent protein (GFP) (*22*), fluorescent chimeras (*23*), OPH (*24*), and scFv antibody (*8*), to the periplasm of *E. coli*. To test whether MPH could be exported by the Tat pathway, we used the twin-arginine signal peptide of trimethylamine *N*-oxide reductase (TorA) from *E. coli*, a molybdopterin-containing protein that is known to be exported by the Tat pathway in *E. coli* (*9*, *25*). A schematic diagram of the TorA−MPH fusion protein is shown in **Figure 1**. A 0.15 kb fragment of *torA* gene encoding the entire signal sequence and the

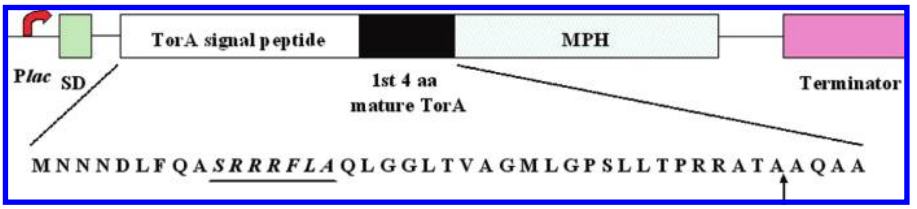

Figure 1. Schematic diagram of the TorA−MPH fusion protein. The *torA−mpd* fusion in pUC18 was constructed as detailed in Materials and Methods. The coding sequence is inserted downstream of a Shine−Dalgarno sequence, and expression is driven from an IPTG-inducible *lac* promoter. The TorA signal peptide is followed by four amino acid residues of the mature TorA protein fused directly to the MPH domain. The twin-arginine motif of "SRRRFLA" is underlined, and the recognition site for type I signal peptidases is indicated by an arrow.

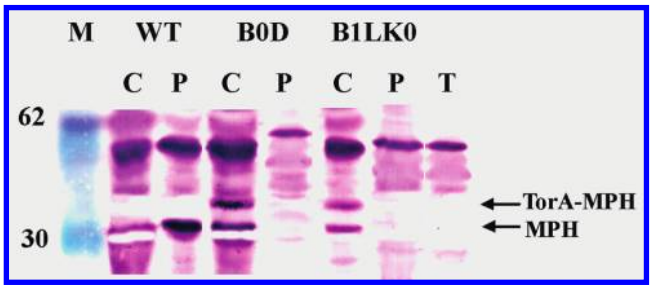

Figure 2. Export of MPH to the periplasm by the Tat pathway in *E. coli* MC4100 transformed with pUTM18. Expression and localization of TorA−MPH were analyzed in transformed MC4100, B0D (like MC4100, △*tatB*) and B1LK0 (like MC4100, △*tatC*) cells after induction with 1 mM IPTG. Cells were fractionated to yield cytoplasmic and periplasmic samples (C and P) and immunoblotted using anti-MPH serum. Mobilities of TorA−MPH and mature-sized MPH are indicated by arrow. Prestained molecular weight markers (M) were used to estimate protein weights. Total cell lysate (T) of MC4100 transformed with pUC18 was used as a negative control.

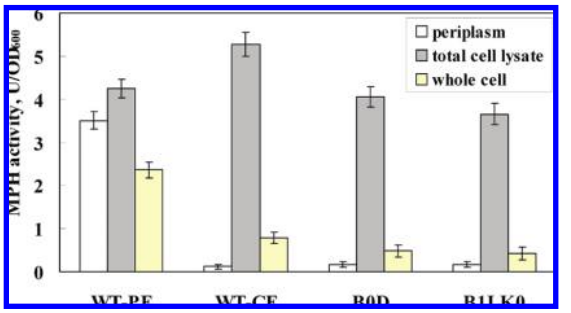

Figure 3. Specific MPH activities of periplasmic fraction, total cell lysate, and whole cell. WT-PE, MC4100 expressing periplasmic MPH; WT-CE, MC4100 expressing cytoplasmic MPH; B0D, Δ*tatB* MC4100; B1LK0, Δ*tatC* MC4100. Cells carrying pUTM18 or pUM18 were incubated at 30 °C for 12 h after induction with 1 mM IPTG. Data are mean values ± standard deviations from three replicates.

first four amino acid residues of the mature TorA was fused in frame with the coding region of MPH. The *torA−mpd* fusion gene was cloned into the pUC18 vector containing the IPTG-inducible *lac* promoter, resulting in the final TorA−MPH construct, pUTM18. The TorA signal peptide contains a conserved AxA motif within the c-region as the recognition site for type I signal peptidases (*10*, *11*). During the release of TorA−MPH into periplasm via membrane-embedded Tat translocase, the precursor was processed to mature-sized MPH by cleaving N-terminal TorA signal peptide.

Export of MPH by the Tat Pathway. Expression of TorA−MPH was induced with 1 mM IPTG, and the localization of MPH was determined by immunoblotting of cytoplasmic and periplasmic fractions from MC4100 cells transformed with pUTM18. This mature-sized MPH (32 kDa) was found predominantly in the periplasmic fraction (**Figure 2**), which indicated that MPH was successfully translocated into the periplasmic space. A considerable amount of this mature-sized MPH was also found in the cytoplasm, possibly resulting from the proteolysis of TorA−MPH. The proteolysis of TorA fusion proteins was observed in previous reports using the Tat pathway for the periplasmic secretion of other proteins (*22*, *24*). To check whether the fractionation was properly performed, Western blot assays were carried out on the same samples using antibodies to β-lactamase, a periplasmic marker and GroEL, a cytoplasmic marker. The data showed that β-lactamase and GroEL were recovered predominantly in the periplasmic and cytoplasmic fractions, respectively, demonstrating that the fractionation protocol was effective (data not shown).

Similar samples were processed from B0D (like MC4100, △*tatB*) and B1LK0 (like MC4100, △*tatC*) cells expressing TorA−MPH. As shown by immunoblotting assays, the expressed TorA−MPH was present as two forms of precursor (37 kDa) and mature-sized MPH (32 kDa) in the cytoplasmic fraction (**Figure 2**). A part of this protein was present as mature-sized MPH in the cytoplasm, which probably reflected the degradation of the chimeric protein. It was reported that the TorA signal peptide may be easily accessible to proteases (*9*, *10*). In *E. coli*, three genes encoding Tat components have been identified as the *tatA*, *tatB* and *tatC* (*25*−*27*). Deletion of *tatB* or *tatC* results in a total block in the export of proteins with twin-arginine signal peptides (*26*, *27*), which indicates that the *tatB* and *tatC* genes are essential Tat pathway components. In this study, the near-complete block in export of the MPH in the *tatB* and *tatC* deletion strains indicated that the export of TorA−MPH was mediated primarily by the Tat system.

The cell lysate activity of *E. coli* MC4100 expressing cytoplasmic MPH was 6.7-fold higher than the whole-cell activity, while the cell lysate activity of MC4100 expressing periplasmic MPH was 1.8-fold higher than the whole-cell activity (**Figure 3**). Even though the cell lysate activity of the cytoplasmic-expressing strain was 1.2-fold higher than that of the periplasmic-expressing strain, the periplasmic-expressing strain showed 3-fold higher whole-cell activity than the control strain expressing cytoplasmic MPH (**Figure 3**). These results indicated that periplasmic secretion of MPH reduced the mass transport limitation of OP pesticides across the cell membrane.

Over 80% of cell lysate activity was detected in the periplasmic fraction of *E. coli* MC4100 cells expressing TorA−MPH (**Figure 3**). In △*tatB* and △*tatC* cells, very little activity was detected in the periplasmic fraction (4% and 5% of cell lysate activity respectively). Only 2% of cell lysate activity was detected in the periplasmic fraction of MC4100 cells transformed with pUM18. These results indicated that MPH was efficiently and functionally exported to the periplasmic space of transformed MC4100 cells.

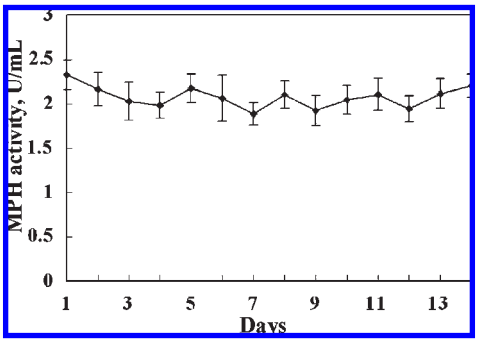

Figure 4. Whole-cell MPH activity in suspended *E. coli* MC4100 cultures expressing periplasmic MPH. The activity was assayed with methyl parathion as substrate. Data are mean values ± standard deviations from three replicates.

To monitor the stability of suspended cultures, whole-cell activity was determined periodically over a 2-week period. As shown in **Figure 4**, whole-cell activity of MC4100 carrying pUTM18 remained at essentially the original level over the 2-week period. The stability of the cells observed here is in line with a previous report using the Tat system for the periplasmic secretion of OPH (*24*). All of these results collectively suggest that the strain expressing periplasmic MPH can be used as a whole-cell biocatalyst for detoxification of OPs.

In Gram-positive bacteria, cells do not possess an additional outer membrane, and cytoplasmic membrane is surrounded by a cell wall. Proteins of up to 25−50 kDa can diffuse freely through the cell wall (*28*). While the proteins are exported across the cytoplasmic membrane, they can be released directly into the culture medium. Extracellular secretion of MPH is advantageous over intracellular production, as it allows the enzymes to freely bind the extracellular substrates. *Bacillus subtilis*, an efficient and safe host for recombinant protein secretion, has been found to possess two distinct Tat translocases (*29*). This strategy of fusing MPH to twin-arginine signal peptide has enormous potential for the extracellular secretion of MPH in *Bacillus subtilis*. This work is currently under investigation.

Biodegradation of Organophosphorus Pesticides by Periplasmic Expression Strain. The degradation rates for four different pesticides were measured using the periplasmic- and cytoplasmic-expressing strains. As shown in **Figure 5**, the periplasmic expression strain carrying pUTM18 degraded these pesticides 3-fold faster than the cytoplasmic expression strain carrying pUM18, which indicated that periplasmic secretion of MPH in *E. coli* improved whole-cell biocatalytic efficiency by reducing the substrate diffusion barrier. *E. coli* is still the most commonly used host for recombinant protein expression because of its fast growth, ease of transformation, and known genetic background. Recombinant *E. coli* strains overexpressing atrazine chlorohydrolase have been applied for *in situ* bioremediation of atrazine-contaminated soil (*30*). We are currently investigating the degradation of organophosphorus compounds in a bioreactor with the *E. coli* strain expressing periplasmic MPH.

At present, OPH-based biocatalysis systems have been developed for detoxification of OPs (*2*, *6*, *7*, *24*). To date, 26 organophosphorus pesticides, which contain dimethyl alkyl groups, are available commercially (*31*). OPH has been shown to lack any hydrolytic activity toward numerous dimethyl OPs (*32*). In contrast, MPH has shown its capability for hydrolyzing numerous dimethyl OPs (*17*). In the U.S., chlorpyrifos accounts for 11% of total pesticide use (*1*). However, chlorpyrifos is hydrolyzed by OPH ~1200-fold less efficiently than paraoxon (*33*). Recently, MPH has shown its ability to hydrolyze

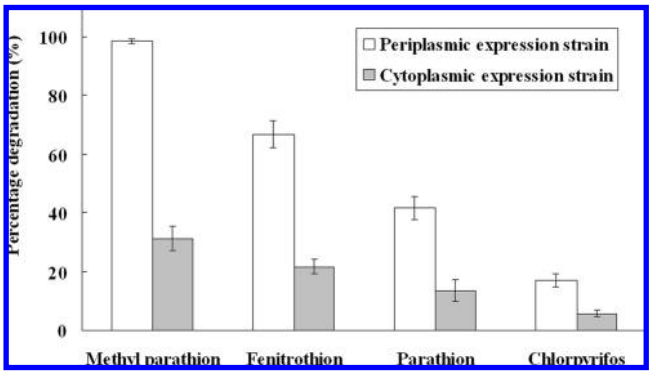

Figure 5. Biodegradation of organophosphorus pesticides by *E. coli* MC4100 cells expressing periplasmic or cytoplasmic MPH. All substrates were added at an initial concentration of 0.4 mM. Data are mean values ± standard deviations from three replicates.

chlorpyrifos (*18*). OPH activity is dependent on divalent cation, while MPH requires no cofactor for maintaining its activity. Therefore, MPH-based biocatalysis systems are advantageous over OPH-based systems for large-scale detoxification of specific OPs.

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