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Biodegradation of Organophosphate Pesticide Using Recombinant Cyanobacteria with Surface- and Intracellular-Expressed Organophosphorus Hydrolase

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The *opd* gene, encoding organophosphorus hydrolase (OPH) from *Flavobacterium* sp. capable of degrading a wide range of organophosphate pesticides, was surface- and intracellular-expressed in *Synechococcus* PCC7942, a prime example of photoautotrophic cyanobacteria. OPH was displayed on the cyanobacterial cell surface using the truncated ice nucleation protein as an anchoring motif. A minor fraction of OPH was displayed onto the outermost surface of cyanobacterial cells, as verified by immunostaining visualized under confocal laser scanning microscopy and OPH activity analysis; however, a substantial fraction of OPH was buried in the cell wall, as demonstrated by proteinase K and lysozyme treatments. The cyanobacterial outer membrane acts as a substrate (paraoxon) diffusion barrier affecting whole-cell biodegradation efficiency. After freeze-thaw treatment, permeabilized whole cells with intracellular-expressed OPH exhibited 14-fold higher bioconversion efficiency (V_{max}/K_m) than that of cells with surface-expressed OPH. As cyanobacteria have simple growth requirements and are inexpensive to maintain, expression of OPH in cyanobacteria may lead to the development of a low-cost and low-maintenance biocatalyst that is useful for detoxification of organophosphate pesticides.

Keywords: Cyanobacteria, surface display, ice nucleation protein, organophosphates, organophosphorus hydrolase, biodegradation

Organophosphorus (OP) compounds are widely used as pesticides (paraoxon, parathion, coumaphos, and diazinon) and chemical warfare agents (sarin and somon). These compounds are potent acetylcholinesterase inhibitors, and various clinical effects can occur from OP poisoning in humans [6]. The growing public concern about their safety and the widespread used of OP throughout the world have stimulated the development of effective and safe remediation strategies for the detoxification of OP.

Organophosphorus hydrolase (OPH), encoded by the *opd* gene of *Pseudomonas diminuta* and *Flavobacterium* sp. [9], is a homodimeric organophosphotriesterase that can hydrolyze a wide range of OPs [8]. The use of *E. coli* whole cells expressing intracellular OPH has been explored as an alternative biological catalyst without the high cost of purifying the enzyme; however, the *E. coli* cell membrane acts as a substrate diffusion barrier, affecting biodegradation efficiency [22]. Attempts have been made to enhance OPH biodegradation efficiency by displaying the OPH onto the cell surface of *E. coli* [23], *Moraxella* sp. [26], and *Saccharomyces cerevisiae* [27].

The ice nucleation protein (Inp) from *Pseudomonas syringae* KCTC1832 (InpK) has been used as an anchoring protein for *E. coli* surface expression of levansucrase [12] and carboxymethylcellulase [11]. The truncated InpK containing only the N- and C-terminal portions (InpNC) is sufficient for targeting OPH to the cell surface of *E. coli* [25] and viral antigens to the cell surface of *Salmonella* [15]. Another related ice nucleation protein (InpV) has also been used for cell surface expression [25]. Although various surface expression systems have been developed for bacteria [24], there is no report of a cyanobacterial surface display system.

Cyanobacteria have been reported to play an important role on bioremediation processes; for examples, removal of the heavy metals from polluted water [7]; biotransformation of mercury (Hg[II]) [16]; and degradation of methyl parathion [1], crude oil [3], aromatic hydrocarbons [20], and xenobiotics [18]. Recombinant filamentous cyanobacteria that degrade organic pollutants have been reported [14]. Because cyanobacteria, free-living photoautotrophic microorganisms, have simple growth requirements and are inexpensive to maintain, the expression of OPH in cyanobacteria may lead to the development of a low-cost and low-maintenance biocatalyst that is useful for detoxification of OP.

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Here, we describe the surface- and intracellular-expressed OPH of the unicellular cyanobacterial cells, *Synechococcus* PCC7942. The OPH was surface displayed using an InpNC anchor. Degradation of OP by the cyanobacterial whole cells expressing OPH was evaluated.

MATERIALS AND METHODS

Strain, Growth Condition, and Plasmid

Synechococcus PCC7942 strain R2-SPc (hereafter, referred to as *Synechococcus*) [13] was grown on BG-11 medium containing 1.5% agar at 30°C under constant illumination of 3,000 lux (*i.e.*, 38 μE/m^2/s). Plasmid pGF101, containing the truncated *inaK* gene (*inaKnc*) of *P. syringae* KCTC 1832 [12], which encodes the 211 and 97 amino acids of the N- and C-terminal domains (InpNC) of ice nucleation protein, respectively, was kindly provided by E. J. Kim (Genefocus, Korea).

Construction of Plasmids for Surface and Intracellular Expressions of OPH

The *opd* gene encoding the truncated OPH without the first 29 amino acids was amplified from the genomic DNA of *Flavobacterium* sp. ATCC27551 using primers opd-F1 and opd-R1 (Table 1) based on the GenBank sequence (M29593). The resulting BamHI/SacI-digested PCR product was cloned into the corresponding sites of pUC18-GUS, which harbors the GUS-(Nos-ter) fragment of pKG [5], to obtain plasmid pUC18-OPH containing *opd*-(*Nos-ter*). For intracellular expression of OPH, the PCR product containing gene cassette P_{tRNA}-*opd* was amplified using primers T1 and opd-R1 with template derived from overlap extension PCR of two PCR products: (i) P_{tRNA} promoter amplified from pKGT-T1R1 [4] using primers T1 and E3-R4; (ii) *opd* gene amplified from pUC18-OPH using primers Lk-opd-F1 and opd-R1. For surface expression of OPH, the PCR product containing gene cassette P_{tRNA}-*inaKnc-opd* was amplified using primers T1 and opd-R1 with template derived from overlap extension PCR of two PCR products: (i) P_{tRNA}-*inaKnc*, which was derived from P_{tRNA} amplified from pKGT-T1R1 [4] using primers T1 and E3-R4, and the *inaKnc* gene amplified from pGF101 using primers Lk-ina-F2 and ina-R2; and (ii) the *opd* gene amplified from pUC18-OPH using primers ina-opd-F2 and opd-R1. The BamHI/SacI-digested PCR products containing gene cassette P_{tRNA}-*opd* or P_{tRNA}-*inaKnc-opd* were cloned into the corresponding sites of pUC18-GUS. The resulting BamHI/EcoRI fragments containing gene cassette P_{tRNA}-*opd*-(Nos-ter) or P_{tRNA}-*inaKnc-opd*-(Nos-ter) were cloned into the corresponding site of shuttle vector pKGT [4] and resulted in pKT-OPH and pKT-InpNC-OPH, respectively. The resulting plasmids were transformed into *Synechococcus* as previously described [13].

OPH Assay

Fresh freeze-thawed whole cells and cell lysate of *Synechococcus* were used to detect the OPH activity. Freeze-thawed whole cells were prepared by freezing the cell pellet at −80°C and thawing before use. To prepare the cell lysate, cells were lysed with a hand-held motor homogenizer in the presence of fine silica. The cell debris and fine silica were removed by centrifugation. Protein concentrations of the cell lysates were determined using the Bio-Rad Detergent Compatible Protein Assay Kit (Bio-Rad Laboratories). For each assay, cell lysate or whole cell (OD_{730}=1) was resuspended in 1 ml of CHES/$CoCl_2$ buffer (50 mM 2-[*N*-cyclohexylamino] ethane-sulfonic acid, pH 9.0, 50 μM $CoCl_2$) containing 2 mM paraoxon (Sigma). Reaction mixtures were incubated at 37°C. OPH activity was measured by following the increase in absorbance of *p*-nitrophenol from the hydrolysis of paraoxon at 400 nm (ε_{400}=17,000/M/cm for *p*-nitrophenol). Specific activities were expressed as units (nanomoles of paraoxon hydrolyzed per minute) per milligram total proteins, or per OD_{730} whole cells. The kinetic parameters, K_m and V_{max}, were determined by fitting the collected data to a Michaelis-Menten equation by nonlinear regression analysis using GraphPad Prism 4.01 software.

Proteinase K and Lysozyme Treatments

For proteinase K treatment, cells were suspended in 1 ml (OD_{730}=1) of 15% sucrose, 15 mM Tris-HCl, 0.1 mM EDTA, pH 7.8, containing 200 μg of proteinase K (US Biological), and then incubated at room temperature for 1 h. For lysozyme treatment, cells were suspended in 1 ml (OD_{730}=1) of 5 mM Tris-HCl, 50 mM NaCl, 5 mM EDTA, pH 8.0, containing 10 μg of lysozyme (Bio Basic), and then incubated at 37°C, 5 min. The treated cells were washed with CHES/$CoCl_2$ buffer and assayed for OPH activity as described above.

Immunostaining CLSM Images

Cells grown for 2 days were harvested and resuspended in phosphate-buffered saline (PBS). Nonspecific staining of antibodies was blocked with 4% bovine serum albumin in PBS. Cells were incubated for 4 h at 4°C with anti-OPH antiserum (dilution 1:200), which was obtained from a mouse injected with partial purified GST-OPH fusion protein. The cells were washed 3 times with PBS containing 0.05% Tween 20 (PBST) and resuspended in PBS with goat anti-mouse IgG conjugated with horseradish peroxidase (dilution 1:500) (Zymed, U.S.A.) and incubated for 2.5 h at 4°C.

Table 1. Primers used in this study.

Primers	Sequence (5'-3')	Target sequence
T1	CGGGATCCTTGCCCTCGCCTCCTAGTCC	P_{tRNA}
E3-R4	CATAAGGGACTGACCACCCGGGGATCGTGACAAGTTACCAATGTAGC	P_{tRNA}
opd-F1	CGGGATCCCTGGATCGATCGGCACAGGCG	*opd*
opd-R1	CGCGAGCTCTCATGACGCCCGCAAGGTCG	*opd*
Lk-opd-F1	TCCCCGGGTGGTCAGTCCCTT/ATGTCGATCGGCACAGGCGATCGG	P_{tRNA}/*opd*
Lk-ina-F2	GATCCCCGGGTGGTCAGTCCCTT/ATGACTCTCGACAAGGCG	P_{tRNA}/*inaK*
ina-R2	CCCGGGCTTTACCTCTATCCAGTC	*inaK*
ina-opd-F2	GACTGGATAGAGGTAAAGCCCGGG/ATGTCGATCGGCACAGGCGATCG	*inaK*/*opd*

Locations of the primers are indicated in Fig. 1. The restriction sites BamHI, SacI, and SmaI are underlined.

Cells were washed 3 times with PBST. Reactivity of immune complexes was visualized by 0.05% diaminobenzidine and 0.1 M imidazole under confocal laser scanning microscopy (CLSM) (Olympus FV1000). Cells, of which primary antibody was omitted, served as negative controls.

Results and Discussion

Optimal OPH Expression of *Synechococcus* Cultures

To investigate the feasibility of *opd* gene expression in cyanobacteria, plasmids pKT-OPH and pKT-InpNC-OPH harboring gene cassettes P_{tRNA}-*opd* and P_{tRNA}-*inaKnc-opd* for intracellular and surface expressions of OPH, respectively, were constructed (Fig. 1) and transformed into *Synechococcus*. Both plasmids contained the cyanobacterial tRNA^{pro} promoter (P_{tRNA}) [4, 5] enabling constitutive expression of OPH.

To determine the optimal OPH expression of *Synechococcus*, OPH activities of freeze-thawed whole cells of cultures harvested at various time courses were performed. The results in Fig. 2 showed that for cells harboring pKT-OPH, the levels of OPH activity were not significantly different in 1-, 2-, and 3-day cultures, and then slightly decreased in 4- and 5-day cultures. For cells harboring pKT-InpNC-OPH, the levels of OPH activity reached a maximum in 2-day culture and decreased thereafter in 3-, 4-, and 5-day cultures. Therefore, further OPH activity determination was performed using the 2-day culture. No OPH activity was detected in

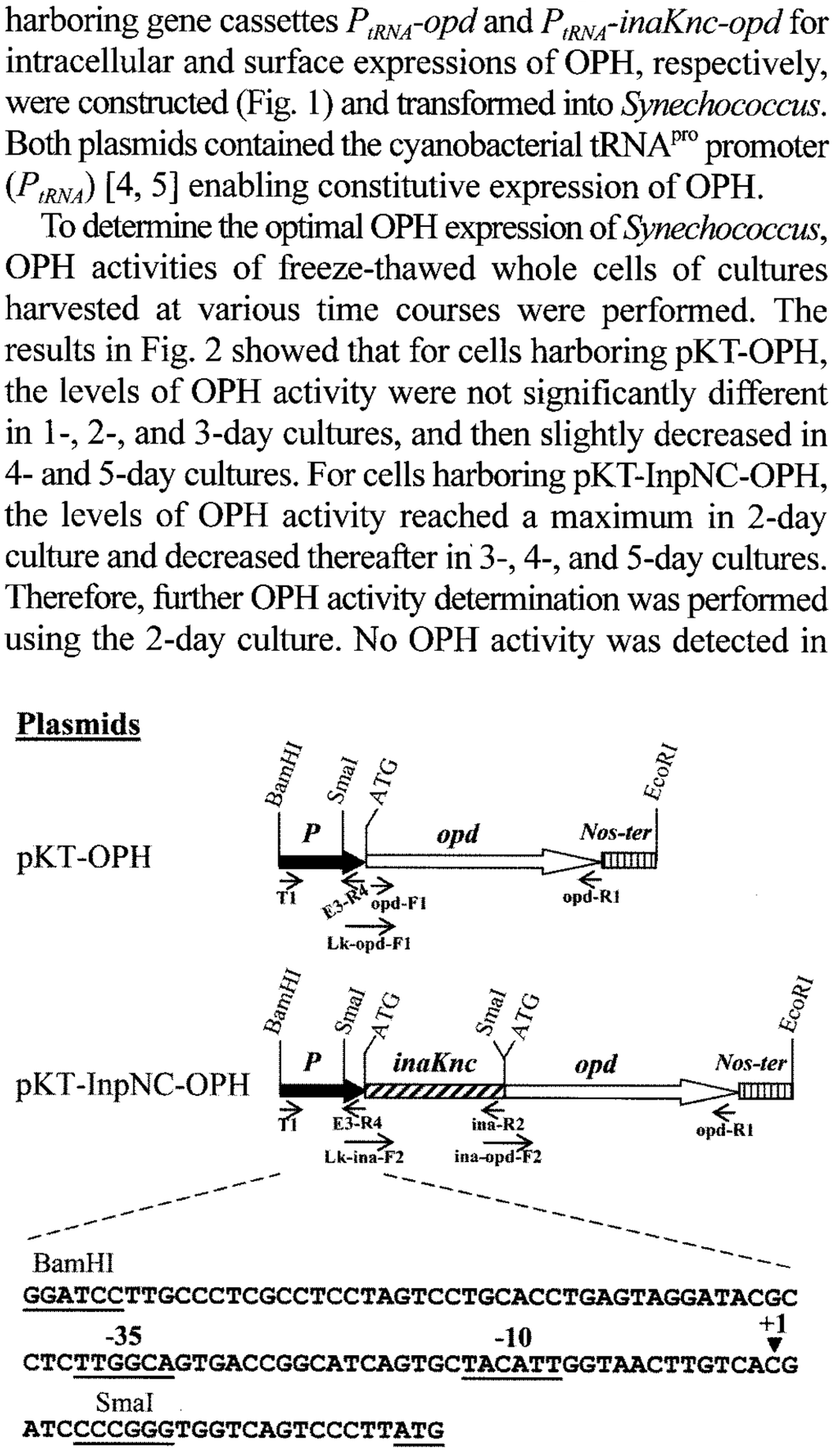

Fig. 1. Plasmids harboring the *opd* gene.
The gene cassettes harboring the P_{tRNA} promoter (P), *inaKnc* gene encoding the truncated ice nucleation protein, *opd* gene encoding organophosphorus hydrolase, and nopaline synthase terminator (Nos-ter) are shown. The gene cassettes were cloned into pKGT plasmid. Locations of primers used in this study are indicated. The P_{tRNA} promoter sequence including the −35 and −10 regions, starting site of mature transcript (+1), and start codon (ATG) are indicated. The figure is not drawn to scale.

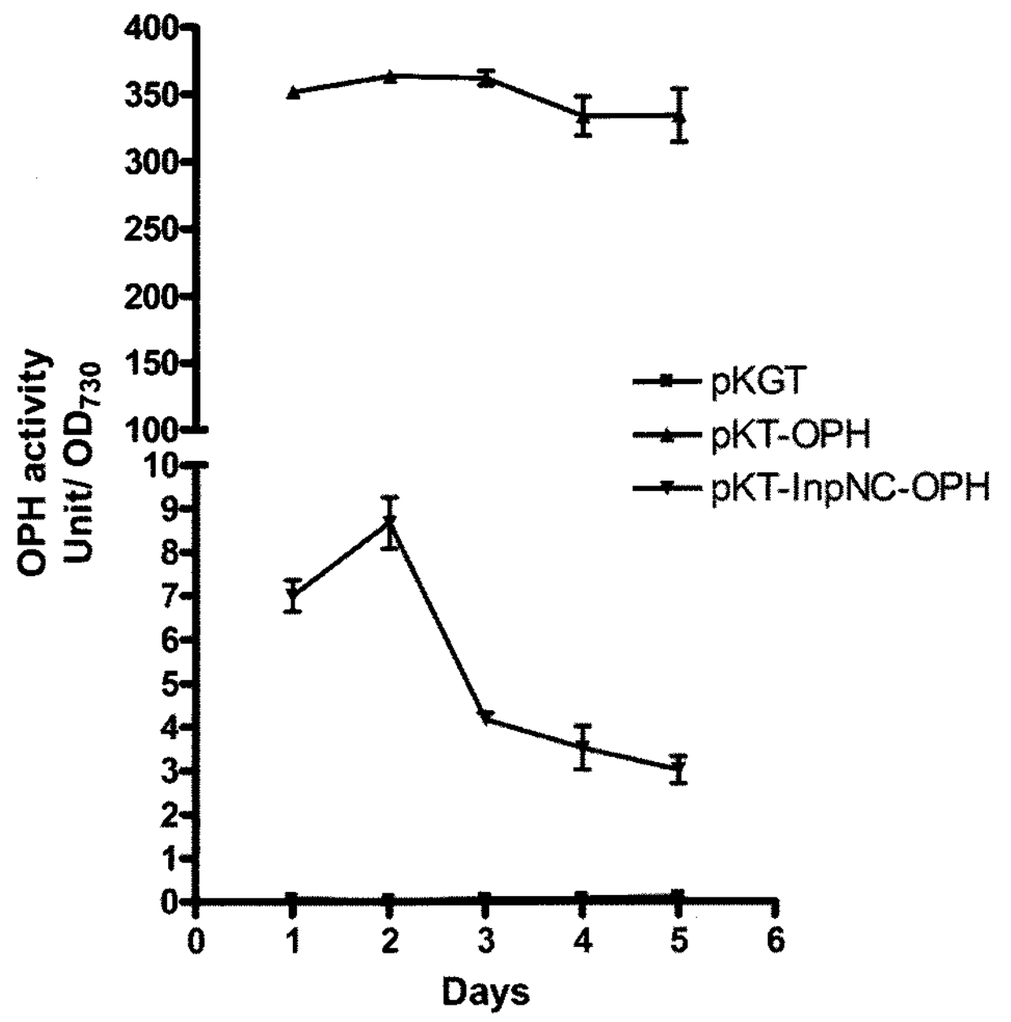

Fig. 2. OPH activities of *Synechococcus* cultures at various time courses.
Cell cultures of *Synechococcus* harboring pKGT, pKT-OPH, and pKT-InpNC-OPH were harvested each day. Freeze-thawed whole-cell suspensions (OD_{730}=1) were assayed for OPH activities. Each value and error bar represent the mean of three independent experiments and its standard deviation.

control cells harboring the pKGT vector. The growth of cells expressing OPH was not significantly different from that of wild type (data not shown).

Probing the Surface Location of OPH Using Immunostaining CLMS Images

The expression of OPH on the surface of *Synechococcus* was verified by immunostaining microscopy. Immunostaining of fresh whole cells was performed by probing with anti-OPH antiserum, followed by incubation with IgG conjugated with horseradish peroxidase as the second antibody. The immunoreactivity of anti-OPH was visualized using diaminobenzidine and amidazole under CLSM. No immunoreactivity of anti-OPH was detected on the surface of cells harboring pKT-OPH (used as control, Fig. 3A), because these antibodies cannot access the intracellular-expressed OPH under the condition employed here. Immunoreactivity of anti-OPH was detected as dark inclusions on the surface of cells harboring pKT-InpNC-OPH (Fig. 3B). It appears that OPH is transported across the membrane and displayed onto the outermost surface of *Synechococcus* using InpNC as an anchoring protein.

Bioconversion Kinetics of Intracellular- and Surface-expressed OPH

The kinetic parameters V_{max} and K_m of intracellular- (pKT-OPH) and surface-expressed OPH (pKT-InpNC-OPH)

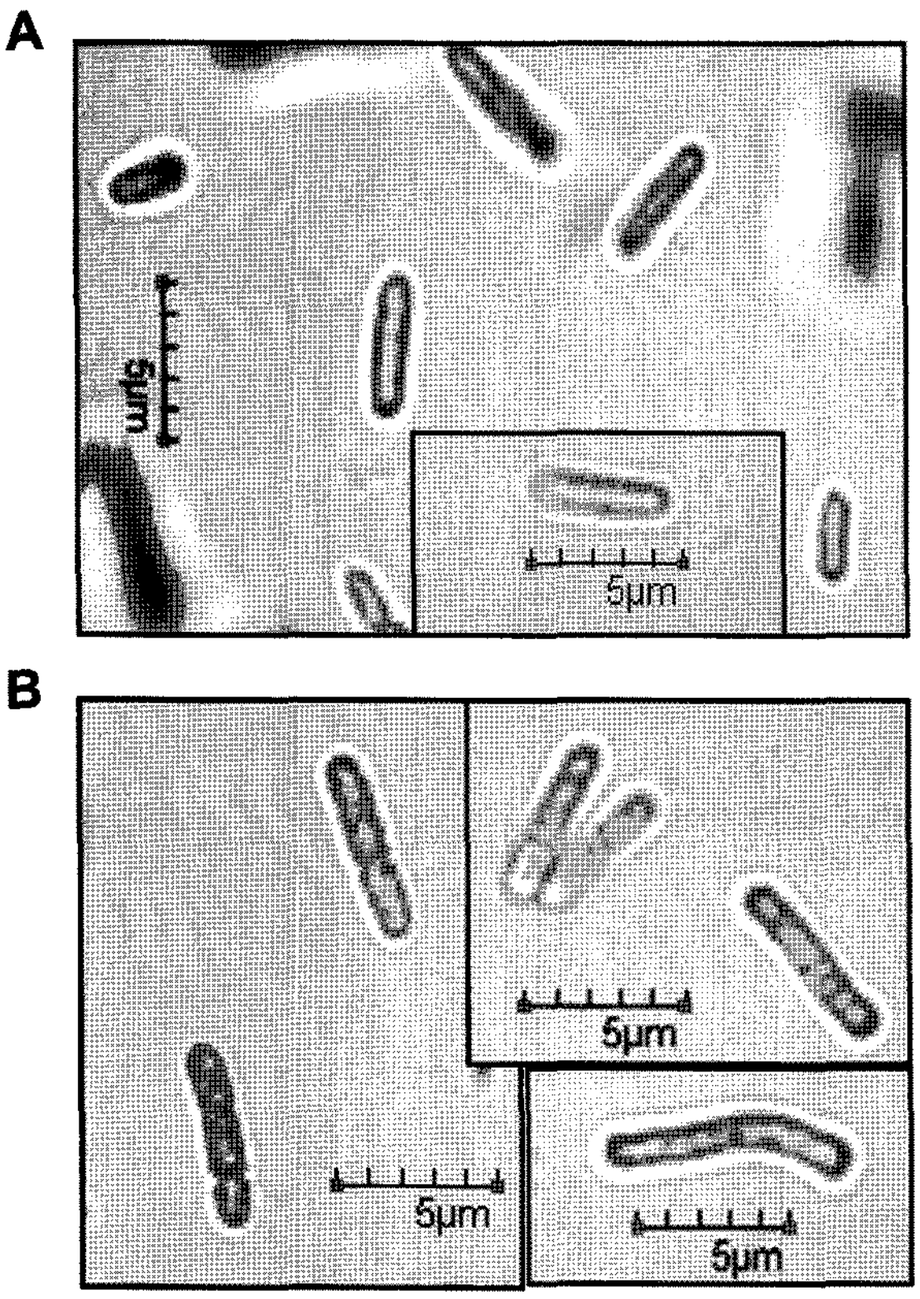

Fig. 3. CLSM images of *Synechococcus* cells expressing OPH.
Synechococcus cells harboring pKT-OPH (**A**) or pKT-InpNC-OPH (**B**). Fresh whole cells were exposed to anti-OPH antiserum and subsequently treated with goat anti-mouse IgG conjugated with horseradish peroxidase. Reactivity of immune complexes was visualized using diaminobenzidine and amidazole under CLSM. Note the immunoreactivity of anti-OPH was detected as dark inclusions on the surface of cells (**B**).

were investigated. The effects of intact and permeabilized outer membrane on OPH activity were also investigated using fresh and freeze-thawed whole cells, respectively. The results are shown in Table 2. The freeze-thawed whole cells with intracellular- and surface-expressed OPH showed 3.5- and 5.5-folds higher V_{max} than that of corresponding fresh whole cells, respectively. The results indicated that the *Synechococcus* outer membrane acts as a substrate (paraoxon) permeability barrier. Permeabilization of the outer membrane of *Synechococcus* caused by freeze-thaw treatment increased OPH activity. Freeze-thaw is known to cause damage to bacterial cell wall and increase outer membrane permeability [2]. The freeze-thawed whole cells with intracellular-expressed OPH showed 43-fold higher V_{max} than that of cells with surface-expressed OPH. Cells with surface-expressed OPH showed 3-fold lower K_m constant than that of cells with intracellular-expressed OPH, implying that the substrate affinity of surface-expressed OPH biocatalyst was higher. The lower K_m constant of surface-expressed OPH might be due to the conformation changes of InpNC-OPH fusion protein. When we calculated the overall reaction efficiency, V_{max}/K_m, the freeze-thawed whole cells with intracellular-expressed OPH exhibited 14-fold higher bioconversion efficiency than that of cells with surface-expressed OPH.

Table 2. Kinetic parameters of OPH for paraoxon.

	pKT-OPH		pKT-InpNC-OPH	
	Fresh	Freeze-thawed	Fresh	Freeze-thawed
V_{max} (μM/min)	138 (±28)	477 (±52)	2.0 (±0.3)	11.0 (±0.7)
K_m (μM)	1,047 (±75)	1,051 (±195)	345 (±121)	347 (±91)
V_{max}/K_m (/min)	0.132	0.454	0.006	0.032

Fresh and freeze-thawed whole cells of *Synechococcus* harboring pKT-OPH and pKT-InpNC-OPH were assayed for OPH activities. The values for kinetic parameters, K_m and V_{max}, were determined using GraphPad Prism 4.01 software. The data are the means of three independent experiments with standard deviations indicated in parentheses.

OPH Activities of Whole Cells Treated with Proteinase K and Lysozyme

Paraoxon degradation was detected in lysate of cells harboring vector pKTG without the *opd* gene (13.9±5.0 units/mg total protein) (Table 3). The results indicated that *Synechococcus* has a natural ability to degrade paraoxon, although at a very low level. It has been shown that cyanobacterium *Anabaena* PCC7120 also has a natural ability to degrade methyl parathion [1]. A similar level of paraoxon degradation (12.2±8.7 units/mg total protein) was detected in lysate of cells with surface-expressed OPH (pKT-InpNC-OPH) (Table 3). Thus, paraoxon degradation in the lysate of surface-expressed OPH was due to the background activity.

To investigate the surface localization of OPH, proteinase K and lysozyme treatments were performed. Proteinase K treatment of intact cells has been used to provide the evidence for the surface location of target proteins [23, 25, 27], since proteinase K cannot readily penetrate through the outer membrane. Lysozyme can damage and penetrate through the outer membrane of *E. coli* resulting in an increase of the outer membrane permeabilization [21, 28]. Lysozyme also destroys bacterial cell walls by cleaving the polysaccharide component of peptidoglycans. For cells with intracellular-expressed OPH (pKT-OPH), the results (Table 3) showed that (i) for untreated cells, the OPH

Table 3. OPH activities of whole cells treated with proteinase K and lysozyme.

Plasmid	Cell lysate[a]	Fresh whole cells[b]			Freeze-thawed whole cells[b]		
		Untreated[c]	Treated with		Untreated[c]	Treated with	
			Proteinase K	Lysozyme		Proteinase K	Lysozyme
pKGT	13.9 (±5.0)	0.1 (±0.1)	0.3 (±0.1)	0.3 (±0.1)	0.2 (±0.1)	0.3 (±0.2)	0.4 (±0.2)
pKT-OPH	688.1 (±66.3)	75.4 (±10.4)	302.5 (±19.4)	387.9 (±44.8)	320.9 (±38.5)	322.2 (±31.4)	368.7 (±57.0)
pKT-InpNC-OPH	12.2 (±8.7)	1.8 (±0.5)	3.2 (±0.3)	5.4 (±0.2)	9.2 (±0.9)	3.9 (±0.4)	2.8 (±0.2)

Fresh and freeze-thawed whole-cell suspensions of *Synechococcus* harboring pKGT, pKT-OPH, and pKT-InpNC-OPH were treated with proteinase K and lysozyme prior to OPH activity assay. The data are the means of three independent experiments, with standard deviations indicated in parentheses.

[a]Unit/mg total protein.

[b]Unit/OD_{730}.

[c]No proteinase K or lysozyme treatment.

activity of freeze-thawed cells was 4.2-fold higher than that of fresh cells; and (ii) after proteinase K or lysozyme treatment, the OPH activities of fresh and freeze-thawed cells were not significantly different. The results suggested that proteinase K, lysozyme, and freeze-thaw treatments resulted in similar increases of outer membrane permeabilization. In the case of cells with surface-expressed OPH (pKT-InpNC-OPH), the results showed that (i) for untreated cells, the OPH activity of freeze-thawed cells was 5-fold higher than that of fresh cells; (ii) OPH activities of proteinase K and lysozyme treated fresh cells were 1.7- and 3-fold higher than that of untreated fresh cells, respectively. The increase of OPH activities might be simply due to the increase of outer membrane permeability caused by proteinase K and lysozyme. (The results suggested that a substantial fraction of OPH was buried in the cell wall.) and (iii) OPH activities of freeze-thawed cells treated with proteinase K and lysozyme decreased by 57% and 69%, respectively. Thus, under the cell wall damage condition caused by freeze-thaw treatment, proteinase K was able to access the buried OPH and lysozyme might cause the loss of cell wall buried proteins including OPH. The results also indicated that proteinase K did not completely degrade the surface-expressed OPH. The Inp is known to be protease resistant, although it is exposed to the outermost surface of the cells [17]. When expressed in *E. coli*, more than 90% of Inp is found in the outer membrane, with 10% in the inner membrane [29]. OPH displayed on the outermost surface of *E. coli* using Inp as an anchoring motif is not completely digested by proteinase K [25]; however, OPH buried in the *E. coli* cell wall has not been evaluated. Our results indicated that a minor fraction of OPH was completely translocated across the membrane and anchored onto the outermost surface of cyanobacterial cells, as verified by immunostaining visualized under CLSM (Fig. 3) and OPH activity analysis (Table 3). However, a substantial fraction of OPH was buried in the cell wall, as demonstrated by proteinase K and lysozyme treatments (Table 3). This may be due to the fact that the cell walls of *E. coli* and cyanobacteria are different. Cyanobacterial cell wall is a combination of Gram-positive and Gram-negative features (*i.e.*, the thick peptidoglycan layer and the outer membrane, respectively). In addition, cyanobacterial outer membranes are normally surrounded by a fibrous sheath and, in many cases, a surface layer [10].

Whole cells with surface-expressed OPH exhibited lower activity than that of cells with intracellular-expressed OPH (Tables 2 and 3), although both plasmids pKT-InpNC-OPH and pKT-OPH containing the *opd* gene under the control of P_{tRNA} promoter were derived from pKGT vector with approximately 30 copies in the cells [19]. It is possible that only properly translocated OPH onto the cell surface could retain functionality. It has been reported that the OPH activity of *E. coli* cells with surface-displayed OPH is higher than that of cells with intracellular-expressed OPH [23]. The OPH activities of *E. coli* cells with surface-displayed OPH using InpK and InpV as an anchoring motif are 15 and 18 units (nmoles per min)/OD_{600}, respectively [25, 27]. In addition, that of *Saccharomyces cerevisiae* using a GPI anchoring system is 130 units/OD_{600} [27]. In this study, the OPH activity of freeze-thawed whole cells with intracellular-expressed OPH (320.9±38.5 units/OD_{730}) was higher than those of whole cells reported so far. The freeze-thaw treatment can be a potential strategy to overcome substrate diffusion limitations in a cyanobacterial whole-cell biocatalyst system. It appears that this system can be successfully used as a whole-cell biocatalyst for detoxification of organophosphate compounds.

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REFERENCES

1. Barton, J. W., T. Kuritz, L. E. O'Connor, C. Y. Ma, M. P. Maskarinec, and B. H. Davison. 2004. Reductive transformation of methyl parathion by the cyanobacterium *Anabaena* sp. strain PCC7120. *Appl. Microbiol. Biotechnol.* **65:** 330–335.
2. Calcott, P. H. and R. A. MacLeod. 1975. The survival of *Escherichia coli* from freeze-thaw damage: Permeability barrier damage and viability. *Can. J. Microbiol.* **21:** 1724–1732.
3. Chaillan, F., M. Gugger, A. Saliot, A. Coute, and J. Oudot. 2006. Role of cyanobacteria in the biodegradation of crude oil by a tropical cyanobacterial mat. *Chemosphere* **62:** 1574–1582.
4. Chungjatupornchai, W., S. Fa-aroonsawat, and S. Panyim. 2002. Characterization of regions of the cyanobacterial tRNA(pro) gene that affect the expression of a beta-glucuronidase reporter gene. *FEMS Microbiol. Lett.* **211:** 57–64.
5. Chungjatupornchai, W., T. Senawong, and S. Panyim. 1999. Isolation and characterization of *Synechococcus* PCC7942 promoters: tRNApro gene functions as a promoter. *Curr. Microbiol.* **38:** 210–216.
6. Donarski, W. J., D. P. Dumas, D. P. Heitmeyer, 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.
7. El-Enany, A. E. and A. A. Issa. 2000. Cyanobacteria as a biosorbent of heavy metals in sewage water. *Environ. Toxicol. Pharmacol.* **8:** 95–101.
8. Grimsley, J. K., J. M. Scholtz, C. N. Pace, and J. R. Wild. 1997. Organophosphorus hydrolase is a remarkably stable enzyme that unfolds through a homodimeric intermediate. *Biochemistry* **36:** 14366–14374.
9. Harper, L. L., C. S. McDaniel, C. E. Miller, and J. R. Wild. 1988. Dissimilar plasmids isolated from *Pseudomonas diminuta* MG and a *Flavobacterium* sp. (ATCC 27551) contain identical *opd* genes. *Appl. Environ. Microbiol.* **54:** 2586–2589.
10. Hoiczyk, E. and A. Hansel. 2000. Cyanobacterial cell walls: News from an unusual prokaryotic envelope. *J. Bacteriol.* **182:** 1191–1199.
11. Jung, H. C., J. H. Park, S. H. Park, J. M. Lebeault, and J. G. Pan. 1998. Expression of carboxymethylcellulase on the surface of *Escherichia coli* using *Pseudomonas syringae* ice nucleation protein. *Enzyme Microb. Technol.* **22:** 348–354.
12. Jung, H. C., J. M. Lebeault, and J. G. Pan. 1998. Surface display of *Zymomonas mobilis* levansucrase by using the ice-nucleation protein of *Pseudomonas syringae*. *Nat. Biotechnol.* **16:** 576–580.
13. Kuhlemeier, C. J., A. A. Thomas, A. van der Ende, R. W. van Leen, W. E. Borrias, C. A. van den Hondel, and G. A. van Arkel. 1983. A host-vector system for gene cloning in the cyanobacterium *Anacystis nidulans* R2. *Plasmid* **10:** 156–163.
14. Kuritz, T. and C. P. Wolk. 1995. Use of filamentous cyanobacteria for biodegradation of organic pollutants. *Appl. Environ. Microbiol.* **61:** 234–238.
15. Lee, J. S., K. S. Shin, J. G. Pan, and C. J. Kim. 2000. Surface-displayed viral antigens on *Salmonella* carrier vaccine. *Nat. Biotechnol.* **18:** 645–648.
16. Lefebvre, D. D., D. Kelly, and K. Budd. 2007. Biotransformation of Hg(II) by cyanobacteria. *Appl. Environ. Microbiol.* **73:** 243–249.
17. Lindow, S. E., E. Lahue, A. G. Govindarajan, N. J. Panopoulos, and D. Gies. 1989. Localization of ice nucleation activity and the *iceC* gene product in *Pseudomonas* syringae and *Escherichia coli*. *Mol. Plant Microbe Interact.* **2:** 262–272.
18. Megharaj, M., K. Venkateswarlu, and A. S. Rao. 1987. Metabolism of monocrotophos and quinalphos by algae isolated from soil. *Bull. Environ. Contam. Toxicol.* **39:** 251–256.
19. Monshupanee, T., S. Fa-aroonsawat, and W. Chungjatupornchai. 2006. A cyanobacterial strain with all chromosomal rRNA operons inactivated: A single nucleotide mutation of 23S rRNA confers temperature-sensitive phenotypes. *Microbiology* **152:** 1417–1425.
20. Narro, M. L., C. E. Cerniglia, C. Van Baalen, and D. T. Gibson. 1992. Metabolism of phenanthrene by the marine cyanobacterium *Agmenellum quadruplicatum* PR-6. *Appl. Environ. Microbiol.* **58:** 1351–1359.
21. Pellegrini, A., U. Thomas, P. Wild, E. Schraner, and R. von Fellenberg. 2000. Effect of lysozyme or modified lysozyme fragments on DNA and RNA synthesis and membrane permeability of *Escherichia coli*. *Microbiol. Res.* **155:** 69–77.
22. Rainina, E. I., E. N. Efremenco, S. D. Varfolomeyev, A. L. Simonian, and J. R. Wild. 1996. The development of a new biosensor based on recombinant *E. coli* for the direct detection of organophosphorus neurotoxins. *Biosens. Bioelectron.* **11:** 991–1000.
23. Richins, R. D., I. Kaneva, A. Mulchandani, and W. Chen. 1997. Biodegradation of organophosphorus pesticides by surface-expressed organophosphorus hydrolase. *Nat. Biotechnol.* **15:** 984–987.
24. Samuelson, P., E. Gunneriusson, P. A. Nygren, and S. Stahl. 2002. Display of proteins on bacteria. *J. Biotechnol.* **96:** 129–154.
25. Shimazu, M., A. Mulchandani, and W. Chen. 2001. Cell surface display of organophosphorus hydrolase using ice nucleation protein. *Biotechnol. Prog.* **17:** 76–80.
26. 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.
27. Takayama, K., S. Suye, K. Kuroda, M. Ueda, T. Kitaguchi, K. Tsuchiyama, T. Fukuda, W. Chen, and A. Mulchandani. 2006. Surface display of organophosphorus hydrolase on *Saccharomyces cerevisiae*. *Biotechnol. Prog.* **22:** 939–943.
28. Wild, P., A. Gabrieli, E. M. Schraner, A. Pellegrini, U. Thomas, P. M. Frederik, M. C. Stuart, and R. Von Fellenberg. 1997. Reevaluation of the effect of lysoyzme on *Escherichia coli* employing ultrarapid freezing followed by cryoelectronmicroscopy or freeze substitution. *Microsc. Res. Tech.* **39:** 297–304.
29. Wolber, P. K., C. A. Deininger, M. W. Southworth, J. Vandekerckhove, M. van Montagu, and G. J. Warren. 1986. Identification and purification of a bacterial ice-nucleation protein. *Proc. Natl. Acad. Sci. USA* **83:** 7256–7260.