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Display of Organophosphorus Hydrolase on the Cyanobacterial Cell Surface Using *Synechococcus* Outer Membrane Protein A as an Anchoring Motif

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Abstract The display of proteins to cyanobacterial cell surface is made complex by combination of Gram-positive and Gram-negative features of cyanobacterial cell wall. Here, we showed that *Synechococcus* outer membrane protein A (SomA) can be used as an anchoring motif for the display of organophosphorus hydrolase (OPH) on cyanobacterial cell surface. The OPH, capable of degrading a wide range of organophosphate pesticides, was fused in frame to the carboxyl-terminus of different cell-surface exposed loops of SomA. Proteinase K accessibility assay and immunostaining visualized under confocal laser scanning microscopy demonstrated that a minor fraction of OPH with 12 histidines fused in frame with the third cell-surface exposed loop of SomA (SomAL3-OPH12H) was displayed onto the outermost cell surface with a substantial fraction buried in the cell wall, whereas OPH fused in frame with the fifth cell-surface exposed loop of SomA (SomAL5-OPH) was successfully translocated across the membrane and completely displayed onto the outermost surface of *Synechococcus*. The successful display of the functional heterologous protein on cell surface provides a useful model for variety of applications in cyanobacteria including screening of polypeptide libraries and whole-cell biocatalysts by immobilizing enzymes.

Keywords Cyanobacteria · *Synechococcus* PCC 7942 · Organophosphorus hydrolase · SomA · Porin · Surface display

Introduction

Cyanobacteria are oxygenic photosynthetic microorganisms. Their cell envelope possesses structural elements typical for both Gram-negative and Gram-positive bacterial envelopes, i.e., outer membrane and plasma membrane separated by a periplasmic space and the thick

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peptidoglycan layer, respectively [1]. In addition, cyanobacteria have an internal photosynthetic membrane system (thylakoids). Targeting of proteins into and across the correct cyanobacterial membrane system remains a great challenge, since the knowledge on this subject is limited. For example, various signal peptides have been used to target the chloramphenical acetyltransferase to periplasmic space and thylakoid lumen of *Synechococcus* PCC 7942 [2]. The Tat pathway has been used to target the green fluorescent protein (GFP) to periplasmic space of *Synechocystis* PCC 6803 [3]. We previously attempted to use the truncated ice nucleation protein (InpNC) from *Pseudomonas syringae* as an anchoring motif to display organophosphorus hydrolase (OPH) and GFP on cell surface of *Synechococcus* PCC 7942. We found that a minor fraction of OPH is displayed onto the outermost cell surface with a substantial fraction buried in the cell wall [4], whereas GFP was found almost entirely in periplasm and not able to display on the outermost cell surface [5]. Various surface expression systems have been developed for bacteria, since display of heterologous proteins has a wide range of biotechnological applications including screening of polypeptide libraries, bioadsorbents for removal of heavy metals, and whole-cell biocatalysts by immobilizing enzymes [6]; however, there is no report of cyanobacterial surface display system.

Synechococcus outer membrane protein A (SomA), a porin, is one of the most abundant proteins of the total envelope proteins of *Synechococcus* PCC 7942 [7]. SomA of *Synechococcus* PCC 7942 showed an overall homology of 97% to that of the closely related strain *Synechococcus* PCC 6301 [8]. The N-terminus of SomA contains a typical signal peptide and a highly conserved surface layer homology (SLH) domain; therefore, it may have a dual function: the formation of diffusion pores as well as act as linkers connecting the outer membrane with the peptidoglycan layer [8].

OPH encoded by the *opd* gene of *Flavobacterium* sp. [9] is a homodimeric organophosphotriesterase that can hydrolyze a wide range of organophosphorus pesticides [10]. OPH has been successfully displayed onto the cell surface of *Escherichia coli* [11], *Moraxella* sp. [12], and *Saccharomyces cerevisiae* [13] to enhance OPH biodegradation efficiency.

In this study, we have used the OPH as a reporter to investigate the ability of SomA to act as an anchoring motif for cyanobacterial surface display system. The OPH was fused in frame to the carboxyl-terminus of different cell-surface exposed loops of SomA. We demonstrated that OPH was successfully translocated across the membrane and completely displayed onto the outermost cyanobacterial cell surface.

Materials and Methods

Strain and Growth Condition

Synechococcus PCC 7942 strain R2-SPc (hereafter, referred to as *Synechococcus*) [14] was grown in liquid or on solid (1.5% agar) BG-11 medium [15] at 30 °C under constant illumination of 3,000 lx (i.e., 38 $\mu E/m^2/s$).

Plasmid Construction

The *somA* gene was amplified from genomic DNA of *Synechococcus* using primers somA-F1 and somA-R2 (Table 1) based on the GenBank sequence (D64077). The resulting BamHI/SalI-digested polymerase chain reaction (PCR) product was cloned into the

Table 1 Primers

Primer	Sequence (5′–3′)	Target sequence
somA-F1	CGGGATCCGAGGGTGGGGTCCGCAAG	Upstream of *somA*
somA-R1	CCGATCGCCTGTGCCGATCGACAT/TGATAAC CTCACACCATGTAGGG	*opd*/*somA*
somA-R2	ACGCGTCGACTCGCCCATCCCTAACTAA	Downstream of *somA*
somA-R3	CCGATCGCCTGTGCCGATCGACAT/GGTAGC ACCAAAAGGCTGGAAGGC	*opd*/*somA*
somA-R6	CCGATCGCCTGTGCCGATCGACAT/GCCACT GAAGAAGGTAGCGTCG	*opd*/*somA*
somA-R7	CCGATCGCCTGTGCCGATCGACAT/GCCCGA GGGATTAAAGCCAGG	*opd*/*somA*
opd-F1	CGGGATCCCTGGATCGATCGGCACAGGCG	*opd*
opd-F2	ATGTCGATCGGCACAGGCGATCGG	*opd*
opd-R5	CGGGATCCTCAATGGTGATGATGGTGATG/TG ACGCCCGCAAGGTCGGTGAC	6His/*opd*
his-R1	CGGGATCCTCAGTGATGGTGATGGTGGTGA TGGTGATGATGGTGATGTGACGC	12His
Nos-R2	CGGGATCCATCTAGTAACATAGATGACACCG	Nos-ter

Locations of the primers are indicated in Fig. 1. The restriction sites BamHI and SalI are *underlined*

corresponding sites of pUC18 to obtain plasmid pUC18-SomA. The *opd6H* gene encoding OPH including six histidines at C-terminus was amplified from pUC18-OPH [4] using primers opd-F1 and opd-R5. The resulting BamHI-digested PCR product was cloned into the BamHI sites of pKTN to obtain pKT-opd6H. Plasmid pKTN and pKTB were derived from shuttle vector pKGT [16] with the deletion of *GUS* gene. For intracellular expression of OPH, the PCR product containing gene cassette *Ps-opd12H* was amplified using primers somA-F1 and his-R1 with template derived from overlap extension PCR of two PCR products: (1) *Ps* promoter amplified from pUC18-SomA using primers somA-F1 and somA-R1 and (2) *opd12H* gene amplified from pKT-opd6H using primers opd-F2 and his-R1. The resulting BamHI-digested PCR product was inserted into BamHI in pKTN to obtain plasmid pPs-opd12H. For surface expression of OPH, the PCR products containing gene cassettes *Ps-L5opd12H*, *Ps-L3opd12H*, and *Ps-L2opd12H* were amplified using primers somA-F1 and his-R1 with template derived from overlap extension PCR of two PCR products: (1) truncated *somA* gene amplified from plasmid pUC18-SomA using primer sets somA-F1 and somA-R3 for Loop 5 (L5), somA-F1 and somA-R6 for L3, and somA-F1 and somA-R7 for L2, and (2) *opd12H* gene amplified from pPs-opd12H using primers opd-F2 and his-R1. The resulting BamHI-digested PCR products were inserted into the corresponding site in pKTN to obtain plasmids pPs-L5opd12H, pPs-L3opd12H, and pPs-L2opd12H, respectively. To construct plasmid pPs-L5opd, the PCR product containing gene cassette *Ps-L5opd* was amplified using primers somA-F1 and Nos-R2 with template derived from overlap extension PCR of two PCR products: (1) *Ps* promoter and truncated *somA* gene amplified from pUC18-SomA using primers somA-F1 and somA-R3, and (2) *opd* gene amplified from pUC18-OPH [4] using primers opd-F2 and Nos-R2. The resulting BamHI-digested PCR product was inserted into the BamHI in pKTB to obtain plasmids pPs-L5opd. The resulting plasmids extracted from *E. coli* were transformed into *Synechococcus* as described [14].

OPH Assay

For each assay, *Synechococcus* fresh whole cells (OD_{730}=1) were 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 substrate (paraoxon) at 400 nm ($\varepsilon_{400} = 17,000/\mathrm{M/cm}$). Specific activities were expressed as units (nanomoles of paraoxon hydrolyzed per minute) per OD_{730} of cells.

$CoCl_2$ Treatment

The 3-day cultures were resuspended (OD_{730}=1) in BG-11 liquid medium containing various concentrations of $CoCl_2$ and further grown for 18 h. The cells were assayed for OPH activity as described above.

Nitrogen Deprivation Treatment

Cells were suspended (OD_{730}=1) in BG-11^N liquid medium in which the ferric ammonia was replaced by ferric citrate or in BG-11^0 liquid medium in which the ferric ammonia and $NaNO_3$ were replaced by ferric citrate and $NaHCO_3$, respectively. The cultures were further grown for 24 h and assayed for OPH activity as described above.

Proteinase K Accessibility Assay

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), then incubated at room temperature for 1 h. The treated cells were assayed for OPH activity as described above.

Immunostaining CLSM Images

Cells grown for 3 days were harvested and resuspended in phosphate-buffer saline (PBS). The procedure for immunostaining of cells was carried out essentially as described [4]. In brief, anti-His monoclonal antibody (GE Healthcare, USA) or anti-OPH antiserum [4] was used as first antibody. Goat anti-mouse IgG conjugated with horseradish peroxidase (Zymed, USA) was used as second antibody. Reactivity of the immune complexes was visualized by 0.05% diaminobenzidine and 0.1 M imidazole under confocal laser scanning microscopy (CLSM; Olympus FV1000). Cells of which first antibody was omitted served as negative controls.

Results and Discussion

Construction of Plasmids Harboring *somA-opd* Fusion Gene

The predicted topology of SomA based on typical features of porins has been reported that the polypeptide might transverse the outer membrane 14 times with the predicted β-strands, which are connected by six short internal loops on the periplasmic side and seven irregular cell-surface exposed loops [8].

In order to investigate the ability of SomA to act as an anchoring motif for the display of OPH onto the cyanobacterial cell surface, plasmids harboring *somA* promoter (*Ps*), N-terminal signal peptide, and truncated *somA* gene fused in frame with the *opd* gene were constructed (Fig. 1). The truncated *somA* gene encoded SomA with C-terminus at amino acid positions 382, 266, and 207 located at the fifth, third, and second cell-surface exposed loop (L5, L3, and L2), respectively. The 12-histidine peptide (12H), a good chelator for the divalent metal ions such as Ni^{2+} [17], was fused in frame with the C-terminus of SomA-OPH fusion protein. The resulting plasmids are pPs-L5opd12H, pPs-L3opd12H, and pPs-L2opd12H encoding fusion proteins SomAL5-OPH12H, SomAL3-OPH12H, and SomAL2-OPH12H, respectively (Fig. 1). In addition, plasmid pPs-L5opd encoding SomAL5-OPH (without 12 H) was constructed. For intracellular expression of OPH, plasmid pPs-opd12H encoding OPH12H was constructed (Fig. 1).

Optimal Expression of SomA-OPH Fusion Protein in *Synechococcus*

To determine the optimal SomA-OPH expression in *Synechococcus*, the OPH activities of cell cultures harvested at various time courses were determined. The results in Fig. 2 showed that for cells harboring pPs-L3opd12H and pPs-opd12H, the levels of OPH activity reached the maximum in 3-day culture. Therefore, further OPH activity determination was performed using 3-day culture.

Catalytically active OPH requires metal ions, such as Co^{2+}, as a cofactor [18]. Addition of a low concentration of cobalt chloride in the growth medium increased OPH activity of recombinant *E. coli* [19] and *S. cerevisiae* [13]. To investigate the effect of $CoCl_2$ on OPH activity of *Synechococcus*, cells were grown in BG-11 liquid medium containing various concentrations of $CoCl_2$. The results in Fig. 3 showed that for cells harboring pPs-L3opd12H

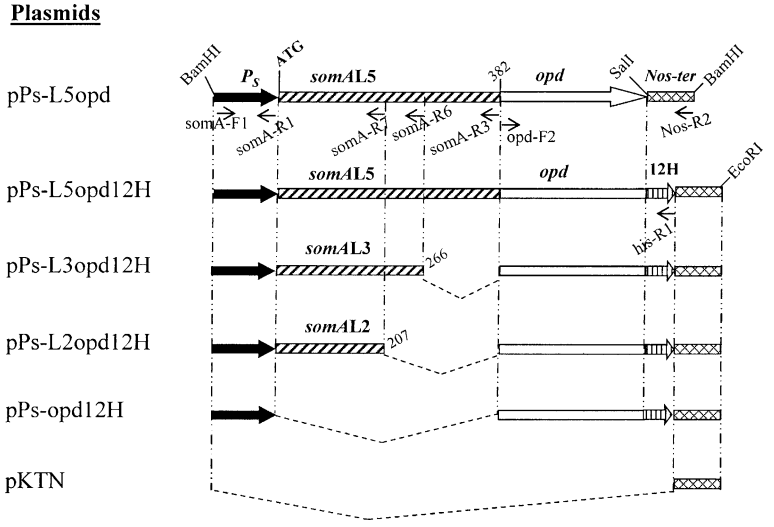

Fig. 1 Constructs encoding OPH. The 3′ end truncated *somA* genes: *somA*L5, L3, and L2 genes (located at the fifth, third, and second cell-surface exposed loop, respectively) were fused in frame with *opd* gene. The numbers of amino acids at the gene fusion positions are with respect to the start codon of SomA. *Ps somA* promoter, *somA* gene encoding *Synechococcus* outer membrane protein A, *opd* gene encoding organophosphorus hydrolase, *12H* 12 histidine peptide, *Nos-ter* nopaline synthase terminator. Locations of primers used in this study are indicated. The figure is not drawn to scale

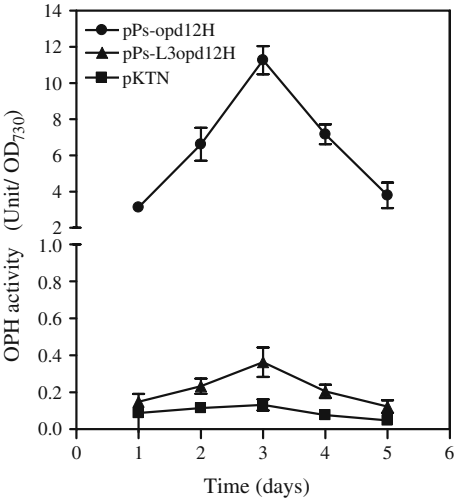

Fig. 2 OPH activities of *Synechococcus* cultures at various time courses. Cell cultures of *Synechococcus* harboring pKTN, pPs-L3opd12H, and pPs-opd12H were harvested each day. Fresh whole-cell suspensions (OD_{730}=1) were assayed for OPH activities. Each value and *error bar* represents the means of three independent experiments and its standard deviation

and pPs-opd12H, the levels of OPH activities reached the maximum at 2 mM $CoCl_2$. Therefore, further OPH activity determination was performed using cells grown in medium containing 2 mM $CoCl_2$.

It has been reported that under the condition of nitrogen deprivation, *somA* promoter is induced, and SomA increases slightly [20]. To investigate the effect of nitrogen deprivation on the expression of *somA–opd* fusion gene under the control of *somA* promoter (*Ps*), the OPH activities of cells grown in medium with or without nitrogen were performed. Figure 4 shows that for the cells harboring pPs-L3opd12H and pPs-opd12H, the level of OPH activity of cells grown in medium supplemented with nitrogen ($BG\text{-}11^N$) was not significantly different from that of corresponding cells grown in nitrogen deprivation medium ($BG\text{-}11^0$). The results indicated that nitrogen deprivation did not affect the expression of *opd* gene. Since the size of *somA* promoter fragment in the gene cassettes is 204 bp (upstream of *somA* start codon), this fragment might not contain the regulatory sequence responding to nitrogen deprivation. The regulatory sequence of the *somA* remains

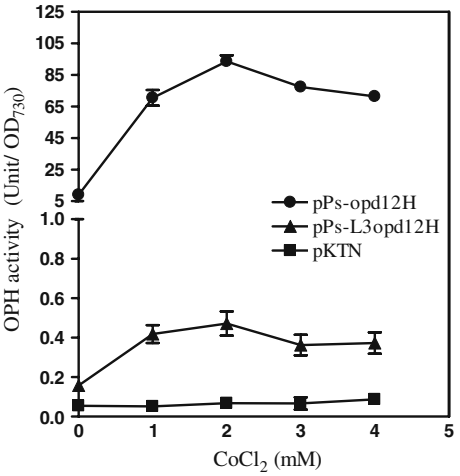

Fig. 3 Effect of $CoCl_2$ on OPH activities of *Synechococcus*. *Synechococcus* harboring pKTN, pPs-L3opd12H, and pPs-opd12H grown in BG-11 liquid medium including various concentration of $CoCl_2$ were harvested. Fresh whole-cell suspensions (OD_{730}=1) were assayed for OPH activities. Each value and *error bar* represents the mean of three independent experiments and its standard deviation

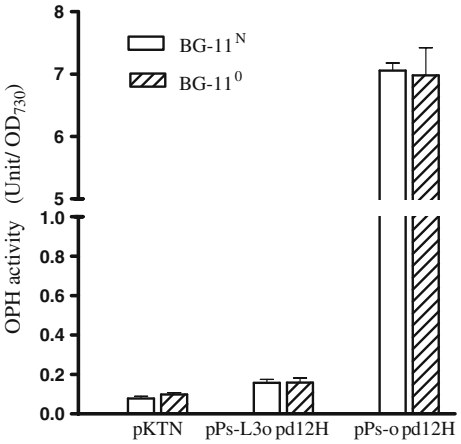

Fig. 4 Effect of nitrogen-depleted medium on OPH activities. *Synechococcus* harboring pKTN, pPs-L3opd12H, and pPs-opd12H were grown in liquid medium BG-11^N (including nitrogen) and BG-11^0 (excluding nitrogen). Whole-cell suspensions (OD_{730}=1) were assayed for OPH activities. Each value and *error bar* represents the mean of three independent experiments and its standard deviation

to be investigated. Therefore, further OPH activity determination was performed using cells grown in medium including nitrogen (BG-11).

Very little background OPH activity was detected in control cells harboring pKTN vector (Figs. 2, 3, 4, and 5). The results agreed well with previous report that *Synechococcus* has natural ability to degrade paraoxon, although at a very low level [4].

Region of SomA Required for OPH Activity of SomA-OPH Fusion Protein

The OPH activities of whole cells harboring the recombinant plasmids were compared. Results in Fig. 5 showed that the OPH activity of cells harboring pPs-L3opd12H was significantly higher than those of pPs-L2opd12H and pPs-L5opd12H. Therefore, the 266 amino acids of N-terminus (SomAL3, Fig. 1) is the optimal length required for OPH activity of the fusion protein. However, we observed that the OPH activity of cells harboring pPs-L5opd12H was 11-fold lower than that of pPs-L5opd (Fig. 5). Therefore, the addition of 12-histidine peptide at the C-terminus of SomAL5-OPH protein decreased the OPH activity.

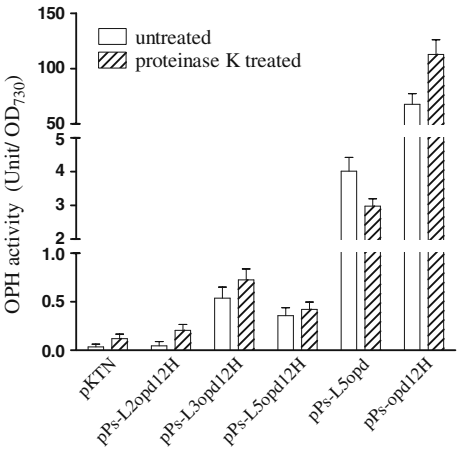

Fig. 5 OPH activities of whole cells treated with proteinase K. *Synechococcus* harboring pKTN, pPs-L2opd12H, pPs-L3opd12H, pPs-L5opd12H, pPs-L5opd, and pPs-opd12H were treated with proteinase K. The proteinase K-treated whole-cell suspensions (OD_{730}=1) were assayed for OPH activities. Each value and *error bar* represents the mean of three independent experiments and its standard deviation

Probing the Surface Location of OPH

Proteinase K accessibility assay of intact cells has been used to provide the evidence for the surface location of OPH [4, 11, 21], since proteinase K can not readily penetrate through the outer membrane. To investigate the surface location of the SomA-OPH fusion protein, proteinase K accessibility assay was performed. The results in Fig. 5 showed that for cells with intracellular-expressed OPH (pPs-opd12H), the OPH activity of proteinase K-treated cells was 1.7-fold higher than that of untreated cells. Similar results were observed in cells harboring pPs-L2opd12H, pPs-L3opd12H, and pPs-L5opd12H that the OPH activity of proteinase K-treated cells was slightly higher than that of corresponding untreated cells (Fig. 5). The results suggested that fusion proteins SomAL2-OPH12H, SomAL3-OPH12H, and SomAL5-OPH12H might not completely display on outermost cell surface. The increases of OPH activities might be simply due to the increases of outer membrane permeability caused by proteinase K. However, the OPH activity of proteinase K-treated cells harboring pPs-L5opd decreased by 25% when compared with that of untreated cells. The results indicated that SomAL5-OPH protein was successfully displayed on the outermost cell surface. Addition of 12H at the C-terminus of SomAL5-OPH protein decreased the OPH activity (pPs-L5opd and pPs-L5opd12H, Fig. 5). It is possible that only properly translocated OPH onto cell surface could retain functionality.

It has been showed that *E. coli* displaying polyhistidine peptides on cell surface can bind to Ni-NTA-agarose beads [17, 22]. In this study, adhersion of 12H to Ni-NTA-agarose beads (Qiagen GmbH, Germany) was performed as described [17] and visualized under phase-contrast microscopy. We found that adhersion of *Synechococcus* harboring pPs-L3opd12H to Ni-NTA-agarose beads was not significantly different from that of control cells with intracellular-expressed OPH (pPs-opd12H; data not shown). Thus, the results also suggested that 12H of SomAL3-OPH12H protein might not completely display on outermost cell surface to access restricted nickel ions. The low expression of SomAL3-OPH12H protein (Figs. 2, 3, and 4) might be another reason.

To determine the OPH activity in cytoplasm of *Synechococcus*, cells were disrupted by French press treatment and centrifuged to obtain the soluble fraction. The OPH activities of the soluble fraction from cells harboring pPs-L3opd12H and pPs-L5opd were barely detected and not significantly different from that of vector pKTN, whereas the OPH activity of soluble fraction from cells with intracellular-expressed OPH (pPs-opd12H) was 44± 10 unit/mg total proteins. Therefore, the OPH of cells harboring pPs-L3opd12H and pPs-L5opd was not located in cytoplasm, implying that the OPH was located in membrane fraction.

Immunostaining CLMS was performed to verify the surface location of the SomAL3-OPH12H and SomAL5-OPH protein. No immunoreactivities of anti-His and anti-OPH were detected on the surface of cells with intracellular-expressed OPH (pPs-opd12H) used as control (Fig. 6b, d, respectively) because these antibodies cannot access the intracellular-expressed OPH under the condition employed here. Immunoreactivities of anti-His and anti-OPH were detected as dark inclusions on the surface of cells harboring pPs-L3opd12H and pPs-L5opd (Fig. 6a, c, respectively). The results indicated that SomAL3-OPH12H and SomAL5-OPH were translocated across the membrane and anchored onto the outermost surface of *Synechococcus*.

Taken together, results from proteinase K accessibility assay (Fig. 5) and immunostaining CLSM (Fig. 6) revealed that (1) a minor fraction of SomAL3-OPH12H was displayed onto the outermost cell surface with a substantial fraction buried in the cell wall. Similar result has been reported for displaying OPH onto the cell surface of *Synechococcus* using InpNC as

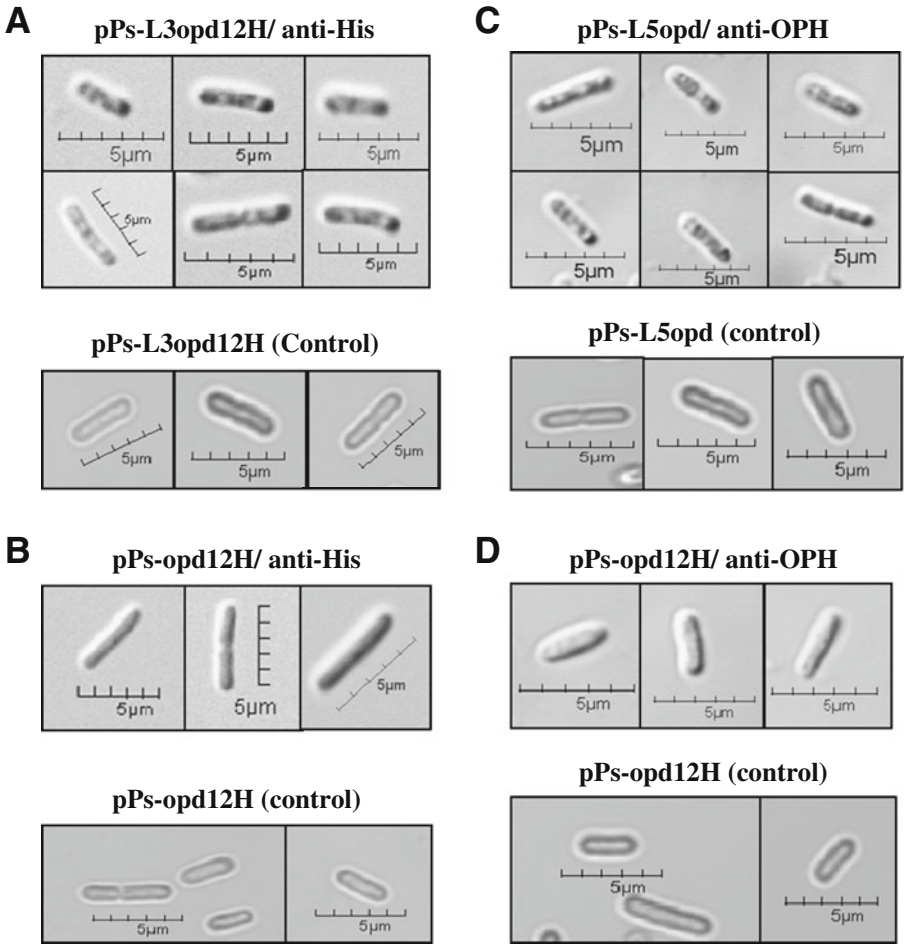

Fig. 6 CLSM images of *Synechococcus* cells expressing OPH. *Synechococcus* harboring plasmids pPs-L3opd12H, pPs-L5opd, and pPs-opd12H were cultured in BG-11 containing 2 mM $CoCl_2$. Whole cells exposed to first antibody, anti-His monoclonal antibody (**a**, **b**), or anti-OPH antiserum (**c**, **d**) are indicated, whereas cells of which first antibody was omitted are indicated as control. The cells were subsequently treated with goat anti-mouse IgG conjugated with horseradish peroxidase. Reactivity of immune complexes was visualized using diaminobenzidine and amidazole under CSLM (Olympus FV1000). The immunoreactivities of anti-His and anti-OPH were detected as dark inclusions on the surface of cells

anchoring motif [4]. (2) Substantial fraction of SomAL5-OPH was translocated across the membrane and completely displayed onto the outermost surface of *Synechococcus*.

Cells with surface-expressed OPH (pPs-L3opd12H and pPs-L5opd) exhibited lower activity than that of cells with intracellular-expressed OPH (pPs-opd12H; Figs. 2, 3, 4, and 5), although all the plasmids containing the *opd* gene under the control of *somA* promoter were derived from pKGT vector. It is possible that only properly translocated OPH onto cell surface could retain functionality. Similar results have been shown in the previous study that the surface-expressed InpNC-OPH has much lower activity than intracellular-expressed OPH [4]. It has been reported that cyanobacteria play an important role on bioremediation processes, for example, removal of the heavy metals from polluted water

[23]; biotransformation of mercury (Hg[II]) [24], and degradation of methyl parathion [25]. Since cyanobacteria, free-living photoautotrophic microorganisms, have simple growth requirements [15] and inexpensive to maintain when compared with *E. coli*, *Pseudomonas*, and *Saccharomyces*, expression of OPH in cyanobacteria may lead to the development of a low-cost and low-maintenance biocatalyst useful for detoxification of OP.

In conclusion, we developed a novel cell surface displaying system in which SomA was used as an anchoring motif for the display of OPH on the cyanobacterial outermost surface. The successful display of the functional heterologous protein on cell surface provides a useful model for variety of applications in cyanobacteria including screening of polypeptide libraries and whole-cell biocatalysts by immobilizing enzymes.

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