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Short Communication

An engineered microorganism can simultaneously detoxify cadmium, chlorpyrifos, and γ-hexachlorocyclohexane

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Many ecosystems are currently co-contaminated with heavy metals such as cadmium (Cd^{2+}) and pesticides such as chlorpyrifos (CP) and γ-hexachlorocyclohexane (γ-HCH). A feasible approach to remediate the combined pollution of heavy metals and pesticides is the use of γ-HCH degrading bacteria endowed with CP hydrolysis and heavy metal biosorption capabilities. In this work, a recombinant microorganism capable of simultaneously detoxifying Cd^{2+}, CP, and γ-HCH was constructed by display of synthetic phytochelatins (EC20) and methyl parathion hydrolase (MPH) fusion protein on the cell surface of the γ-HCH degrading *Sphingobium japonicum* UT26 using the truncated ice nucleation protein (INPNC) as an anchoring motif. The surface localization of INPNC–EC20–MPH was verified by cell fractionation, Western blot analysis, immunofluorescence microscopy, and proteinase accessibility experiment. Expression of EC20 on the cell surface not only improved Cd^{2+} binding but also alleviated the cellular toxicity of Cd^{2+}. As expected, the rates of CP and γ-HCH degradation were reduced in the presence of Cd^{2+} for cells without EC20 expression. However, expression of EC20 (higher Cd^{2+} accumulation) significantly restored the levels of CP and γ-HCH degradation. These results demonstrated that surface display of EC20 enhanced not only Cd^{2+} accumulation but also protected the recombinant strain against the toxic effects of Cd^{2+} on CP and γ-HCH degradation.

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Introduction

γ-Hexachlorocyclohexane (γ-HCH, also called lindane), which is a known organochlorine insecticide, is very stable in the environment and persists in many decades because it is difficult for γ-HCH to be degraded by microorganisms [1]. Although γ-HCH has been prohibited for use in developed countries, it is still being used for household pest control and forest management in China.

Chlorpyrifos (CP) is a broad-spectrum, moderately toxic organophosphate insecticide that is widely being used to control various pests in agriculture. Chlorpyrifos irreversibly inhibits acetylcholine esterase in the central nerve system, leading to the accumulation of acetylcholine and a subsequent loss of nerve function [2].

Cadmium (Cd) is of great concern to environmental safety because of its toxicity and intrinsically non-degradable nature. Cadmium tends to accumulate in soil through adsorption and poses a serious health threat to humans such as itai-itai disease or cancers [3].

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The toxicity of one pollutant can be potentiated by the presence of other pollutants. For example, cadmium and chlorpyrifos often co-exist in the same site, and organisms are exposed to them simultaneously, leading to joint toxicity such as synergistic hepatoxicity [4]. Co-exposure to organophosphate and organochlorine insecticides can potentiate the toxicity of the individual compounds [5]. Therefore, the combined pollution of heavy metals and organics needs to be resolved by developing new techniques.

Synthetic phytochelatins (EC20), which are protein analogs of phytochelatins (PCs), have a high cadmium-binding capacity because of their repetitive metal-binding motif $(Glu–Cys)_nGly$ [6]. Methyl parathion hydrolase (MPH), which is encoded by the *mpd* gene from *Stenotrophomonas* sp. YC-1, has high hydrolytic activity against chlorpyrifos [7].

The cell envelope of Gram-negative bacteria consists of a cytoplasmic membrane, cell wall, and outer membrane. Chlorpyrifos and cadmium cannot easily pass through the outer membrane; thus, they have no access to MPH or EC20 residing within the cell. A clever solution is to display MPH or EC20 onto the cell surface, overcoming the rate-limiting step in the detoxification of chlorpyrifos or cadmium by natural isolates expressing MPH or EC20 intracellularly. Currently, MPH or EC20 has been displayed on the surface of bacterial cells for enhanced hydrolysis of chlorpyrifos and improved biosorption of cadmium [8, 9].

Many ecosystems are currently co-contaminated with pesticides and heavy metals, such as γ-HCH, chlorpyrifos and cadmium. A promising strategy to remediate the combined pollution of cadmium, chlorpyrifos, and γ-HCH is the use of γ-HCH-degrading bacteria endowed with chlorpyrifos hydrolysis and cadmium biosorption capabilities. *Sphingobium japonicum* UT26 is a well-characterized γ-HCH degrading bacterium that utilizes γ-HCH as the sole source of carbon and energy for its growth under aerobic conditions [10]. The aerobic degradation pathway of γ-HCH in strain UT26 has been extensively researched in the past two decades. In this study, EC20–MPH fusion protein was functionally displayed on the cell surface of *S. japonicum* UT26 using the truncated ice nucleation protein (INPNC) as an anchoring motif, resulting in a multifunctional strain with the capability to simultaneously detoxify cadmium, chlorpyrifos, and γ-HCH.

Materials and methods

Bacterial strains, plasmids, and culture conditions

S. japonicum UT26, which was isolated from an upland experimental field in Japan where γ-HCH had been applied once a year for 12 years [10], was used as host strain for construction of a multifunctional pollutant-detoxifying microorganism. An *inpnc–ec20–mpd* fusion gene was chemically synthesized by BGI Inc., Beijing, China, and then ligated into pUC57-simple vector. The synthetic gene was released from pUC57-simple with *Eco*RI and *Hin*dIII, and subcloned into similarly digested *E. coli–Pseudomonas* shuttle vector pVLT33 [11] to create pVINPEM. The surface expression vector, pVINPEM, coding for INPNC–EC20–MPH was introduced into *S. japonicum* UT26 using the electroporation method [12] and used to target EC20–MPH onto the cell surface of strain UT26. The constructs pINCM and pCPM [9] were used, respectively, for expressing INPNC–MPH and cytoplasmic MPH.

S. japonicum UT26 harboring pVINPEM was grown at 30 °C in 1/3 Luria-Bertani (LB) medium [13] or minimal salt medium (MSM) [14] supplemented with kanamycin to a final concentration of 50 $\mu g\,ml^{-1}$. Expression of INPNC–EC20–MPH fusion protein was induced with 0.5 mM IPTG for 24 h at 30 °C when cells were grown to an OD_{600} of 0.5.

Cell fractionation and Western blot analysis

Cell fractionation was performed according to the method of Shimazu et al. [15]. Samples of total cell lysate, soluble fraction, and outer membrane fraction were analyzed by 12% SDS-PAGE [13] and electro-blotted using a semidry transfer system (Bio-Rad) onto a PVDF membrane (Roche). The membrane was blocked for 1 h in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) supplemented with 3% BSA. The membrane was then incubated with rabbit polyclonal anti-MPH antibody at a dilution of 1:500 for 2 h, washed with TBST for 30 min, and incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Abcam) at a dilution of 1:2000 for 1 h. Immunoreactive bands were detected by enhanced chemiluminescence using an ECL Plus kit (Amersham).

Immunofluorescence microscopy

Cells harboring pVINPEM were incubated in PBS buffer (pH 7.4) containing 1% BSA for 1 h prior to immunolabeling. Subsequently, cells were incubated with polyclonal anti-MPH antibody at a dilution of 1:500 overnight at 4 °C. After five washes with PBS buffer, the cells were reacted with goat anti-rabbit IgG conjugated with rhodamine (Abcam) at a dilution of 1:64 for 3 h at room temperature. Finally, cells were washed five times with PBS buffer and observed by a fluorescence microscopy (Olympus).

Proteinase accessibility assay

Cells harboring pVINPEM were centrifuged and resuspended ($OD_{600} = 1.0$) in 1 ml of 15% sucrose, 15 mM Tris–HC1, and 0.1 mM EDTA, pH 7.8. Samples were incubated for 1 h with 5 μl of 20 mg/ml proteinase K at room temperature. Proteinase K treated cells were assayed for Western blot and immunofluorescence microscopy as described above.

MPH activity assay

Cells harboring pVINPEM were cultured and induced as described above. Then, the cells were washed with 100 mM phosphate buffer (pH 7.4) and diluted to an $OD_{600} = 1.0$ with the same buffer. MPH activity assay mixtures (1 ml, 3% methanol) contained 50 μg ml^{-1} chlorpyrifos (added from a 10 mg ml^{-1} methanol stock solution), 870 μl of 100 mM phosphate buffer (pH 7.4), and 100 μl of cells ($OD_{600} = 1.0$). Activities are expressed as units (1 μmol of chlorpyrifos hydrolyzed per minute) per OD_{600} whole cells.

Cadmium binding experiments

Overnight cultures of strain UT26 harboring pVINPEM were inoculated into 20 ml of 1/3 LB medium containing 50 μg ml^{-1} kanamycin in 250-ml flasks and grown at 30 °C and 200 rpm in a shaker. When the culture OD_{600} reached 0.5, 0.5 mM IPTG, and 80 μM $CdCl_2$ (final concentrations) were added to each flask. Subsequently, 1-ml samples were taken at 6, 12, and 24 h after induction for cadmium-binding analyses. The cell density of the samples was determined at each time point. Whole-cell binding of cadmium was determined by atomic absorption spectrometry. Triplicate samples from independent flasks were taken for each data point.

Overnight cultures were inoculated into 1/3 LB medium containing 50 μg ml^{-1} kanamycin and induced with 0.5 mM IPTG at mid-exponential growth phase. Cells were grown for 24 h, iced for 20 min, and centrifuged at 3000 rpm at 4 °C for 30 s. The cell pellet was washed with 10 mM PBS buffer (pH 7.4) and resuspended to an OD_{600} of 1.0 in the same buffer. $CdCl_2$ (80 μM) was added to the cell suspensions, and samples were taken at 0, 20, 60, 120, and 180 min. Triplicate samples were taken for each time point. Whole-cell binding of cadmium was determined by atomic absorption spectrometry.

For whole-cell Cd^{2+} content, cells were washed three times with 5 mM HEPES buffer (pH 7.1) containing 0.8% NaCl. The washed pellets were dried in an oven set to 65 °C for 24 h and digested with 100 μl of concentrated nitric acid for at least 48 h. The total Cd^{2+} content was measured using atomic absorption spectroscopy (AA-7000, Shimadzu, Japan) in the flame mode. The data were normalized to the number of nanomoles of cadmium per milligram of dry cell weight.

Biodegradation experiments

Degradation of chlorpyrifos and γ-HCH by the recombinant strain UT26 was studied. The recombinant strain UT26 harboring pVINPEM was precultured at 30 °C in 1/3 LB medium containing 50 μg ml^{-1} kanamycin. The bacterial cells were harvested during log phase, washed twice with MSM, and resuspended in MSM. The cell suspensions were used as inoculums. Subsequently, 10^6 cells/ml lere inoculated into 50 ml of MSM supplemented with 50 μg ml^{-1} kanamycin, 0.5 mM IPTG, 0.1% glucose, 50 μg ml^{-1} chlorpyrifos, and 10 μg ml^{-1} γ-HCH. The cultures were maintained in 250-ml flasks at 30 °C and 200 rpm in a shaker. Samples were taken at regular time intervals for pesticide residue analyses. Both the samples inoculated with wild-type strain UT26 and uninoculated samples were kept as the controls.

The toxic effects of Cd^{2+} on chlorpyrifos and γ-HCH degradation were also investigated. Cells harboring pVINPEM were cultured in 1/3 LB medium containing 50 μg ml^{-1} kanamycin. When the OD_{600} reached 0.5, expression of INPNC–EC20–MPH was induced with 0.5 mM IPTG and $CdCl_2$ was added to a final concentration of 80 μM. After induction for 24 h at 30 °C, cells were harvested by centrifugation and resuspended to an OD_{600} of 1.0 in PBS buffer. The cell suspensions were incubated with 50 μg ml^{-1} chlorpyrifos or 10 μg ml^{-1} γ-HCH in the presence of 80 μM $CdCl_2$ at 30 °C and 200 rpm in a shaker. Samples were taken every 1 h to measure the amount of chlorpyrifos or γ-HCH remaining. Analyses for pesticide residues were performed using a Hewlett-Packard 5890 II GC equipped with an ECD detector and a capillary HP-1 column as described previously [14].

Results and discussion

Surface localization of INPNC−EC20−MPH in *S. japonicum* UT26

In the present study, to investigate the feasibility of targeting EC20–MPH fusion protein onto the cell surface of *S. japonicum* UT26, the truncated InaV protein (INPNC) [16] was used as a surface anchoring motif. For expression of INPNC–EC20–MPH, the *inpnc–ec20–mpd* fusion gene was subcloned into pVLT33, a medium-copy-number vector, to give pVINPEM. The broad-host-range vector, pVLT33, is an RSF1010 derivative and therefore is able to replicate in a wide variety of

Gram-negative bacteria [11]. Expression of INPNC–EC20–MPH was tightly regulated by a *tac* promoter due to the presence of the *lacI*^{q} gene on the plasmid. The nucleotide sequence of the *inpnc–ec20–mpd* fusion gene and its corresponding amino acid sequence are shown in Supporting Information Fig. S1.

Expression of INPNC–EC20–MPH in *S. japonicum* UT26 harboring pVINPEM was verified by Western blot analysis with polyclonal anti-MPH antibody. A specific protein band was observed from total cell lysate at the position of ~70 kDa, which matched well with the molecular mass estimated from the deduced amino acid sequence of the fusion protein (Fig. 1). As expected, no target protein was detected with total cell lysate of wild-type strain UT26. Furthermore, the localization of INPNC–EC20–MPH in the outer membrane fraction was demonstrated by immunoblotting of subcellular fractionated samples with polyclonal anti-MPH antibody. The vast majority of target proteins were present in the outer membrane fraction. In contrast, very few target proteins were found in the soluble fraction (Fig. 1).

Immunolabeling with specific antibodies or antisera is a useful technique to detect proteins displayed on the cell surface [17]. To verify the anchorage of INPNC–EC20–MPH on the outer membrane, cells were reacted with rabbit polyclonal anti-MPH antibody and then fluorescently stained with rhodamine-conjugated goat anti-rabbit IgG antibody. Since the outer membrane acting as a permeability barrier prevents antibodies from interacting with intracellular target proteins, only surface-displayed proteins can be labeled with antibodies. When observed by a fluorescence microscopy, the orange fluorescence was observed on the cells harboring pVINPEM, while the control cells expressing a cytoplasmic MPH (pCPM) were not immunostained (data not shown).

A proteinase accessibility assay was also used for further demonstration of the surface localization of EC20–MPH. Since proteases cannot diffuse through the outer membrane, only surface-displayed proteins can be degraded by proteases [18]. The outer membrane fraction isolated from proteinase K-treated cells was probed with polyclonal anti-MPH antibody. As expected, no desired proteins were found in the outer membrane fraction, indicating that the EC20–MPH displayed on the cell surface was completely degraded by proteinase K.

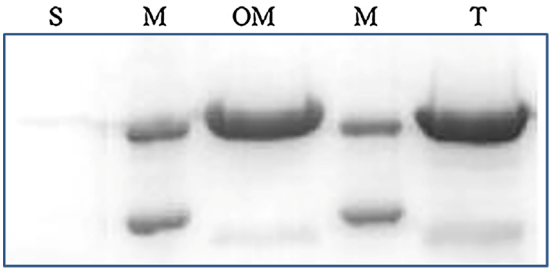

Figure 1. Western blot analysis for the expression and subcellular localization of INPNC–EC20–MPH fusion protein in *S. japonicum* UT26 harboring pVINPEM. Lanes: T, total cell lysate; OM, outer membrane fraction; S, soluble fraction; M, protein markers. Anti-MPH polyclonal antibody was used at a 1:500 dilution.

Proteinase K-treated cells were immunolabeled with polyclonal anti-MPH antibody and rhodamine-conjugated IgG secondary antibody. As a result, these cells were not immunostained completely, indicating that the EC20–MPH exposed on the cell surface had been removed by proteinase K. From all of these results, we concluded that EC20–MPH was displayed on the cell surface using the INPNC anchor.

Simultaneous degradation of γ-HCH and chlorpyrifos by recombinant strain UT26

The surface-displayed MPH directly interacts with the extracellular substrates, which enhances whole-cell catalytic activity. Moreover, the N-terminus of the MPH is linked to the outer membrane via the INPNC anchor, so the enzyme is immobilized with enhanced structural stability. Prior to IPTG induction, the MPH activity was not detected. The whole-cell MPH activity increased gradually after induction with 0.5 mM IPTG and reached a maximum (0.182 U/OD_{600}) at 24 h.

The γ-HCH (10 μg ml^{-1}) was completely degraded by the recombinant strain UT26 within 32 h. The recombinant strain UT26 degraded γ-HCH as fast as wild-type strain UT26, indicating that surface display of INPNC–EC20–MPH did not affect the intrinsic γ-HCH degradation capability of wild-type strain UT26. Chlorpyrifos (50 μg ml^{-1}) was completely degraded by the recombinant strain UT26 in 24 h. In contrast, the concentration of chlorpyrifos did not change in the culture of wild-type strain UT26. Moreover, the recombinant strain UT26 could completely degrade 50 μg ml^{-1} chlorpyrifos and 10 μg ml^{-1} γ-HCH in MSM in the presence of 80 μM $CdCl_2$ within 30 and 42 h, respectively. The recombinant strain accumulated 3.61 nmol Cd^{2+}/mg (dry weight) of cells at 42 h. These results highlighted the potential of the recombinant strain for use in the detoxification of a mixture of cadmium, chlorpyrifos, and γ-HCH.

Enhanced cadmium binding and cell growth by surface-displayed EC20

Experiments were performed with growing cultures to investigate the functionality of EC20 in whole-cell binding of Cd^{2+}. Cultures of *S. japonicum* UT26 carrying pVINPEM and the control strain carrying pVLT33 were

grown in the presence of 80 μM $CdCl_2$, and whole-cell binding of Cd^{2+} was determined at 6, 12, and 24 h postinduction. The recombinant strain UT26 with surface-expressed EC20 (pVINPEM) accumulated a significantly higher amount of Cd^{2+} than the control strain carrying pVLT33. Surface display of EC20 enabled the recombinant strain carrying pVINPEM to bind 3.82 nmol Cd^{2+}/mg (dry weight) of cells, a level 12-fold higher than that of the control strain carrying pVLT33 at 24 h (Fig. 2A).

Cadmium-binding experiments were also performed with resting cultures in the presence of 80 μM $CdCl_2$. Resting cells of *S. japonicum* UT26 carrying pVINPEM bound cadmium rapidly, with 88% of maximum binding occurring with the first 60 min (Fig. 2B). This rapid initial binding rate suggests an instantaneous binding of Cd^{2+} by the surface-displayed EC20, followed by slower nonspecific binding to other cell surface components. Consistent with the result obtained with growing cultures, the maximum cadmium content of 6.62 nmol Cd^{2+}/mg (dry weight) of cells for *S. japonicum* UT26 carrying pVINPEM was fivefold higher than that of the control strain carrying pVLT33, 1.45 nmol Cd^{2+}/mg (dry weight) of cells, suggesting that the enhanced binding effect by the EC20 moiety was sustained even under slow-growing conditions.

More importantly, surface display of EC20 also conferred increased cadmium resistance to the recombinant strain. The final cell density for *S. japonicum* UT26 harboring pVINPEM was twofold higher than the control strain carrying pVLT33 grown in the presence of 80 μM $CdCl_2$ (Fig. 3). Moreover, *S. japonicum* UT26 carrying pVINPEM grown in the presence of cadmium reached a final cell density similar to that of the control strain carrying pVLT33 grown in the absence of cadmium.

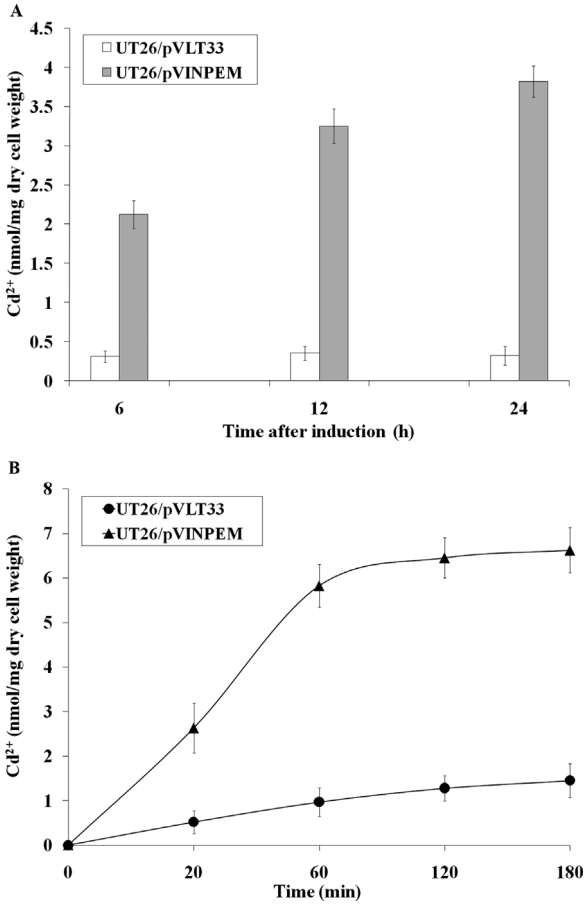

Figure 2. Cadmium-binding experiments with growing and resting cells of *S. japonicum* UT26 harboring pVINPEC20. (A) Cadmium accumulation from growing cultures of *S. japonicum* UT26 harboring pVINPEC20 in the presence of 80 μM of $CdCl_2$. When the culture OD_{600} reached 0.5, expression of INPNC–EC20–MPH was induced with 0.5 mM IPTG, and $CdCl_2$ was added to a final concentration of 80 μM. (B) Cadmium accumulation from resting cells of *S. japonicum* UT26 harboring pVINPEC20. Resting cells were resuspended to an $OD_{600} = 1.0$ in 10 mM PBS buffer (pH 7.4) containing 80 μM of $CdCl_2$ and incubated for 3 h at 30 °C and 200 rpm in a shaker. Cells harboring pVLT33 were used as the negative control. Data are mean values ± standard deviations from three replicates.

Restoration of chlorpyrifos and γ-HCH degradation by surface-displayed EC20

To investigate the effect of cadmium on chlorpyrifos and γ-HCH degradation, *S. japonicum* UT26 harboring pVINPEM was grown in the presence or absence of $CdCl_2$. Then, cells were harvested, resuspended to an $OD_{600} = 1.0$ in PBS buffer and incubated with 50 μg ml^{-1} chlorpyrifos or 10 μg ml^{-1} γ-HCH in the presence of

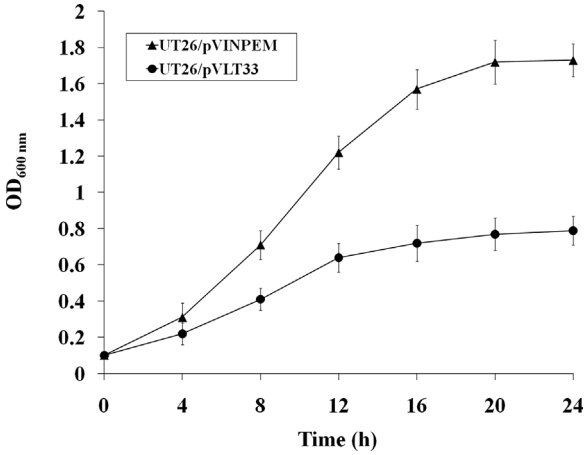

Figure 3. Growth curves of *S. japonicum* UT26 harboring pVLT33 or pVINPEM in the presence of 80 μM of $CdCl_2$. Cells were grown in 1/3 LB medium containing 50 μg ml^{-1} kanamycin, 0.5 mM IPTG, and 80 μM of $CdCl_2$. Cells were inoculated at $OD_{600} = 0.1$ into 1/3 LB medium. Data are mean values ± standard deviations from three replicates.

$CdCl_2$. The rate of chlorpyrifos or γ-HCH degradation was determined by measuring their remaining amount.

In the presence of 80 μM Cd^{2+}, the rate of γ-HCH degradation was decreased by 69% for the recombinant strain without EC20 expression (Fig. 4A). Similarly, the chlorpyrifos degradation rate was significantly impaired (up to 61%) in the presence of 80 μM Cd^{2+} (Fig. 4B). These results demonstrated that the presence of cadmium was detrimental to γ-HCH and chlorpyrifos degradation. In comparison, the rates of γ-HCH and chlorpyrifos degradation were significantly restored for the recombinant strain with surface-expressed EC20. These results indicated that the surface-expressed EC20 moiety could effectively sequester Cd^{2+} ions, minimizing transport, and the inhibitory effect of intracellular Cd^{2+} on γ-HCH and chlorpyrifos degradation.

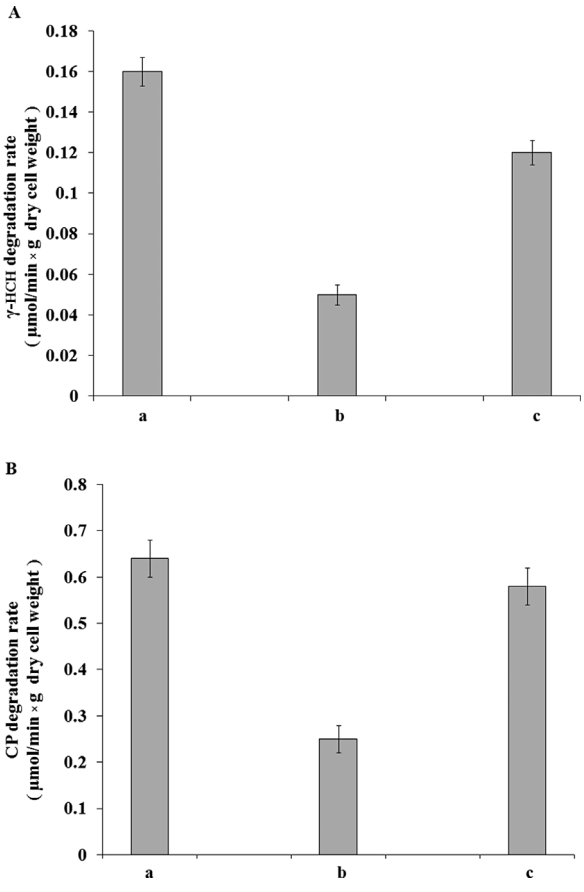

Figure 4. (A) The γ-HCH degradation rate for *S. japonicum* UT26 harboring pVINPEM incubated with 80 μM $CdCl_2$. (a) No induction and no Cd^{2+} added, (b) Cd^{2+} added but no induction, and (c) Cd^{2+} added and induction for EC20 expression. (B) The chlorpyrifos degradation rate for *S. japonicum* UT26 harboring pVINPEM or pINCM incubated with 80 μM $CdCl_2$. (a) Induction for EC20–MPH expression (pVINPEM) and no Cd^{2+} added, (b) Cd^{2+} added and induction for MPH expression (pINCM), and (c) Cd^{2+} added and induction for EC20–MPH expression (pVINPEM). Data are mean values ± standard deviations from three replicates.

Conclusion

The engineered strain displaying EC20–MPH fusion protein was endowed with the ability to simultaneously detoxify γ-HCH, chlorpyrifos, and cadmium. Moreover, the toxic effects of cadmium on degradation of γ-HCH and chlorpyrifos were reduced greatly due to improved cadmium accumulation. Therefore, the engineered strain could potentially be applied for simultaneous detoxification of heavy metals and pesticides.

Acknowledgments

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