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ORIGINAL PAPER

Engineering chlorpyrifos-degrading *Stenotrophomonas* sp. YC-1 for heavy metal accumulation and enhanced chlorpyrifos degradation

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Abstract Many ecosystems are currently co-contaminated with pesticides and heavy metals, such as chlorpyrifos and cadmium. A promising strategy to remediate mixed chlorpyrifos-cadmium-contaminated sites is the use of chlorpyrifos-degrading bacteria endowed with cadmium removal capabilities. In this work, a gene coding for synthetic phytochelatins (EC20) with high cadmium-binding capacity was introduced into a chlorpyrifos-degrading bacterium, *Stenotrophomonas* sp. YC-1, resulting in an engineered strain with both cadmium accumulation and chlorpyrifos degradation capabilities. To improve the cadmium-binding efficiency of whole cells, EC20 was displayed on the cell surface of *Stenotrophomonas* sp. YC-1 using the truncated ice nucleation protein (INPNC) anchor. The surface localization of the INPNC-EC20 fusion protein was demonstrated by cell fractionation, Western blot analysis, and immunofluorescence microscopy. Expression of EC20 on the cell surface not only improved cadmium binding, but also alleviated the cellular toxicity of cadmium. As expected, the chlorpyrifos degradation rate was reduced in the presence of cadmium for cells without EC20 expression. However, expression of EC20 (higher cadmium accumulation) completely restored the level of chlorpyrifos degradation. These results demonstrated that EC20 expression not only enhanced cadmium accumulation, but also reduced the toxic effect of cadmium on chlorpyrifos degradation.

Keywords Cadmium · Phytochelatins · Chlorpyrifos · *Stenotrophomonas*

Introduction

The coexistence of various pollutants in the environment and food chains is very concerning because of the combined impact the on environment and public

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health, especially if simultaneous exposure of organisms to pollutants can potentiate the toxicity of individual components and the joint toxicity of pollutants poses adverse health effects to humans (Kortenkamp et al. 2007). For example, cadmium (Cd) and chlorpyrifos (CP) are often present together in the same environmental media and food chains, and organisms are exposed to them simultaneously, leading to joint toxicity such as synergistic hepatoxicity (Mansour et al. 2009; Chen et al. 2013).

Cadmium has broad industrial applications, e.g., in battery production and electroplating, and it is substantially dispersed in the environment. Exposure to cadmium can cause serious diseases, such as itai-itai disease or even cancers (Alkorta et al. 2004). Cadmium can enter farmland by sewage irrigation if cadmium-containing industrial wastewater is secretly discharged into rivers, which leads to cadmium contamination of soil. Chlorpyrifos is a broad-spectrum, moderately toxic organophosphate insecticide that is increasingly being used in agriculture. Chlorpyrifos irreversibly inhibits acetylcholine degradation in the human body, leading to a subsequent loss of nerve function and eventual death (Singh and Walker 2006; Singh 2009). Farmland is often co-contaminated with chlorpyrifos and cadmium.

Cadmium cannot be degraded by microorganisms. Synthetic phytochelatins (ECs), which are protein analogs of phytochelatins (PCs), have a high cadmium-binding capacity because of their repeating Glu–Cys moieties (Bae and Mehra 1997). The use of microorganisms producing ECs has been shown to be a promising technique for the efficient biosorption of cadmium. However, biosorption of cadmium by intracellular ECs has been problematic because of the limited cadmium uptake. A clever solution to bypass this problem is to directly anchor the ECs onto the cell surface. Currently, ECs have been displayed on the surface of bacterial cells for improved biosorption of cadmium. Both *Escherichia coli* and *Moraxella* sp. strains with EC20 (20 cysteines) displayed on the cell surface using the Lpp–OmpA fusion system or truncated ice nucleation protein (INPNC) anchor accumulated a substantially higher amount of cadmium than the wild-type cells (Bae et al. 2000, 2002).

To date, several chlorpyrifos-degrading bacteria have been isolated from different genera such as *Enterobacter* strain B-14 (Singh et al. 2004), *Alcaligenes faecalis* DSP3 (Yang et al. 2005), *Stenotrophomonas* sp. YC-1 (Yang et al. 2006), *Sphingomonas* sp. Dsp-2 (Li et al. 2007), *Paracoccus* sp. TRP (Xu et al. 2008), *Bacillus pumilus* C2A1 (Anwar et al. 2009), and *Cupriavidus* sp. DT-1 (Lu et al. 2013). It is well known that the presence of heavy metals can inhibit a broad range of microbial processes including metabolism, growth, and aerobic biodegradation of a variety of organic pollutants (Said and Lewis 1991; Sandrin and Maier 2003). Since organic pollutants such as chlorpyrifos and cadmium may occur simultaneously in a contaminated environment, cadmium may inhibit the activity of chlorpyrifos-degrading bacteria. However, microorganisms with both cadmium biosorption and chlorpyrifos degradation capabilities have not yet been isolated from the natural environment. Therefore, the creation of engineered microorganisms will be crucial to remediate soils co-contaminated with chlorpyrifos and cadmium.

We previously isolated chlorpyrifos-degrading *Stenotrophomonas* sp. YC-1; this strain contains a chromosome-based *mpd* gene that encodes methyl parathion hydrolase (MPH), which is responsible for hydrolysis of chlorpyrifos to 3,5,6-trichloro-2-pyridinol (TCP) (Yang et al. 2006). In this work, EC20 was displayed onto the cell surface of *Stenotrophomonas* sp. YC-1 using an INPNC anchor, resulting in an engineered strain with both cadmium biosorption and chlorpyrifos degradation capabilities.

Materials and methods

Bacterial strains, plasmids, and culture conditions

Stenotrophomonas sp. strain YC-1 isolated from activated sludge by selective enrichment with chlorpyrifos (Yang et al. 2006) was used as host strain for cell surface display of synthetic phytochelatins (EC20). A surface expression vector, pVINPEC20, coding for INPNC-EC20 fusion protein was used to target EC20 onto the cell surface. A fusion gene coding for ice nucleation protein-synthetic phytochelatin fusion protein (INPNC-EC20) was chemically synthesized and then subcloned into *Eco*RI/*Hin*dIII-digested pVLT33 (de Lorenzo et al. 1993), an *E. coli*–*Pseudomonas* shuttle vector, to generate pVINPEC20. Transformation of pVINPEC20 into *Stenotrophomonas* sp. YC-1 was performed using the electroporation method of Garg et al. (1999).

Strains bearing plasmids were grown at 30 °C in Luria-Bertani (LB) medium (Sambrook and Russel 2001) supplemented with kanamycin to a final concentration of 50 μg/ml. Expression of INPNC-EC20 fusion protein was induced with 1 mM IPTG for 24 h at 30 °C when cells were grown to an OD_{600} of 0.5.

Cell fractionation

Cells were harvested and resuspended in 25 mM Tris-HCl buffer (pH 8.0). After disruption of the cells by sonication and a brief clarifying spin, the clarified lysate was ultracentrifuged at 50,000 rpm for 1 h at 4 °C, and the supernatant was retained as the soluble fraction. The pellet (total membrane fraction) was resuspended with PBS containing 0.01 mM $MgCl_2$ and 2 % Triton X-100 for solubilizing the inner membrane and was incubated for 30 min at room temperature; then the outer membrane fraction was repelleted by ultracentrifugation (Li et al. 2004).

SDS-PAGE and Western blot analysis

Subcellular fractionated samples were mixed with sample buffer (200 mM Tris-HCl, pH 6.8, 8 % SDS, 0.04 % bromophenol blue, 8 % β-mercaptoethanol, 40 % glycerol), boiled for 5 min, and analyzed by 12 % SDS-PAGE (Sambrook and Russel 2001). Proteins were electroblotted onto nitrocellulose membranes using a tank transfer system (Bio-Rad) at 40 V for 3 h. Blotted membranes were blocked in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05 % Tween-20) with 3 % BSA. The membrane was incubated with rabbit anti-INPNC polyclonal serum at a 1:1,000 dilution at 30 °C for 2 h and washed with TBST. Subsequently, the membrane was allowed to react with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Promega) at a 1:1,000 dilution at 30 °C for 1 h. The membrane was then stained with NBT/BCIP (Novagen) for visualizing antigen-antibody conjugates.

Immunofluorescence microscopy

Cells were harvested and washed with PBS buffer (pH 7.4), then resuspended in PBS containing 3 % BSA ($OD_{600} = 0.5$) for blocking at 30 °C for 1 h. Cells were then incubated with rabbit anti-INPNC serum diluted (1:500) in PBS + BSA solution at 30 °C for 2 h. After being washed five times with PBS, the cell-antibody complex was incubated with goat anti-rabbit IgG antibody conjugated with rhodamine (Invitrogen) at a dilution of 1:100 at 30 °C for 1 h. Finally, cells were washed five times with PBS and observed by fluorescence microscopy (Olympus).

Cadmium-binding experiments

Cells carrying pVINPEC20 were inoculated into 20 ml of LB medium containing 50 μg/ml kanamycin at an initial $OD_{600} = 0.02$. When the OD_{600} value of the culture reached 0.5, expression of INPNC-EC20 fusion protein was induced with 1 mM IPTG, and $CdCl_2$ was added to a final concentration of 16 μM. Samples were withdrawn at various culture times to determine whole cell accumulation of cadmium.

IPTG-induced cells were washed twice with 10 mM PBS buffer (pH 7.0) and resuspended to an OD_{600} of 1.0 in the same buffer supplemented with $CdCl_2$ to a final concentration of 16 μM. The resting cells were incubated for 2 h on a shaker at 200 rpm and 30 °C. Samples were removed at 0, 5, 30, 60, and 120 min to determine whole-cell binding of Cd^{2+}. In the cadmium-binding experiments, cells carrying pVLT33 were used as the control.

For whole-cell Cd^{2+} content, cells were washed with 5 mM HEPES buffer (pH 7.1) containing 0.8 % NaCl for three times. 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 adsorption 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.

Chlorpyrifos degradation experiments

Stenotrophomonas sp. YC-1 harboring pVINPEC20 was grown overnight at 200 rpm and 30 °C in LB medium containing 50 μg/ml kanamycin and 0.4 mM chorpyrifos in either the presence or absence of $CdCl_2$ and IPTG. Cells were harvested and resuspended to an OD_{600} of 1.0 in PBS buffer. Resting cells were then incubated with 0.4 mM chlorpyrifos at 200 rpm and 30 °C, and the rate of chlorpyrifos degradation was

determined in either the presence or absence of $CdCl_2$. Samples were taken every 10 min to measure the amount of chlorpyrifos remaining.

Chlorpyrifos in liquid samples was extracted with dichloromethane. The extracts were dried over anhydrous Na_2SO_4, and the solvent was allowed to evaporate at room temperature. The residual was dissolved in methanol, and 20 μl of the resulting solution was analyzed by reversed-phase HPLC (600 Controller, Rheodyne 7725i Manual Injector and 247 Dual λ Absorbance Detector; Waters Co., Milford, MA, USA). The separation column (4.6 mm × 250 mm × 5 μm) for the HPLC was filled with Kromasil 100-5C18. The mobile phase containing methanol/water (80:20, v/v) was delivered at a flow rate of 0.8 ml/min. The detection wavelength was 230 nm. The concentration of chlorpyrifos was determined by comparison with values in a calibration curve established for concentrations between 0.5 and 150 mg/l.

Results

Surface expression of EC20 using an INP anchor in *Stenotrophomonas* sp. YC-1

INP and the truncated version of INP containing only the N- and C-terminal portions have been used to target a wide range of monomeric and dimeric proteins onto the cell surface of gram-negative bacteria without any adverse effects on cell growth and integrity (Salem et al. 2008). In this study, the truncated InaV protein (INPNC) (Schmid et al. 1997) was used to target EC20 onto the cell surface of *Stenotrophomonas* sp. YC-1. A schematic diagram of the cell surface display of INPNC-EC20 fusion protein is shown in Supplementary Material. The synthetic *inpnc-ec20* fusion gene was subcloned into a medium-copy-number and broad-host-range vector, pVLT33, which is an RSF1010 derivative and therefore is able to replicate in a wide variety of gram-negative bacteria (de Lorenzo et al. 1993) to give pVINPEC20. Expression of INPNC-EC20 fusion protein was tightly regulated by a *tac* promoter because of the presence of the *lacI*^{q} gene on the plasmid. The nucleotide sequence of the *inpnc–ec20* fusion gene and its corresponding amino acids are shown in the Supplementary Material.

To verify the synthesis of INPNC-EC20 fusion protein in *Stenotrophomonas* sp. YC-1, Western blot analysis was performed with whole-cell lysate of strain YC-1 carrying pVINPEC20 after induction with 1 mM IPTG. A 37-kDa protein band that corresponds to the size of the INPNC-EC20 fusion protein was detected from cells carrying pVINPEC20. However, no such protein was detected with control cells carrying the empty vector pVLT33. The localization of INPNC-EC20 fusion protein in the outer membrane was demonstrated by immunoblotting of different subcellular fractions with anti-INPNC serum. The results of Western blotting are shown in the Supplementary Material.

Immunolabeling with specific antibodies or antisera is a useful tool to detect surface-exposed proteins (Li et al. 2004). To investigate whether INPNC-EC20 fusion protein was displayed correctly on the cell surface in a stable conformation, immunofluorescence microscopy was used. Cells were probed with rabbit anti-INPNC serum as a primary antibody and then fluorescently stained with rhodamine-labeled goat anti-rabbit IgG antibody. Since antibodies cannot diffuse through the outer membrane, the specific interactions should only occur with proteins exposed on the cell surface. When observed with a fluorescence microscopy, cells carrying pVINPEC20 were brightly fluorescent, while control cells carrying pVLT33 were not immunostained at all (data not shown). These results indicated that the cell surface was covered with antibody-rhodamine complex, which confirmed that INPNC-EC20 fusion protein was successfully displayed on the cell surface.

Enhanced cadmium binding and cell growth by surface-expressed EC20

Experiments were performed with growing cultures to investigate the functionality of INPNC-EC20 fusion protein in whole-cell binding of Cd^{2+}. The cells carrying pVINPEC20 and the control cells carrying pVLT33 were grown in the presence of 16 μM $CdCl_2$, and whole-cell binding of Cd^{2+} was determined at 6, 12, and 24 h postinduction. These results indicated that cells expressing EC20 on the cell surface (pVINPEC20) accumulated a significantly higher amount of Cd^{2+} than control cells carrying pVLT33. Expression of INPNC–EC20 fusion protein enabled the cell to bind 7.12 nmol Cd^{2+}/mg (dry weight) of cells, and the Cd^{2+} binding capacity of the cell was 12-fold higher than that of the control cell at 24 h (Fig. 1a).

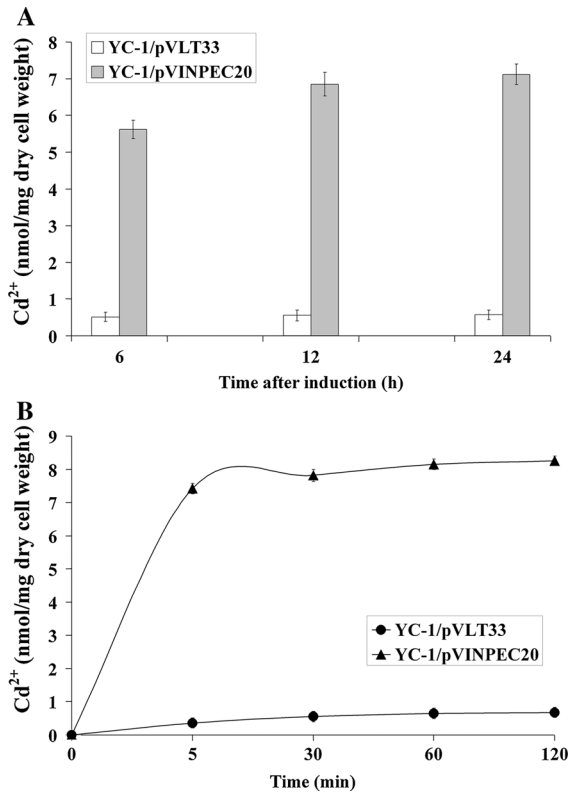

Fig. 1 Cadmium-binding experiments with growing and resting cells of *Stenotrophomonas* sp. YC-1 harboring pVINPEC20. **a** Cadmium accumulation from growing cultures of *Stenotrophomonas* sp. YC-1 harboring pVINPEC20 in the presence of 16 μM of $CdCl_2$. Cells were inoculated into 20 ml of LB medium containing 50 μg/ml kanamycin at an initial $OD_{600} = 0.02$. When the OD_{600} reached 0.5, expression of INPNC–EC20 fusion protein was induced with 1 mM IPTG, and $CdCl_2$ was added to a final concentration of 16 μM. **b** Cadmium accumulation from resting cells of *Stenotrophomonas* sp. YC-1 harboring pVINPEC20. Resting cells were resuspended to an $OD_{600} = 1.0$ in 10 mM PBS buffer (pH 7.0) containing 16 μM of $CdCl_2$ and incubated for 2 h at 200 rpm and 30 °C. Cells harboring pVLT33 were used as control. Data are mean values ± standard deviations from three replicates

Cadmium-binding experiments were also performed with resting cultures in the presence of 16 μM $CdCl_2$. Resting cells of the engineered strain carrying pVINPEC20 bound cadmium rapidly, with 90 % of maximum binding occurring within the first 5 min (Fig. 1b). This rapid initial binding rate suggests an instantaneous binding of Cd^{2+} by the surface-exposed EC20, followed by slower nonspecific binding to other cell surface components. The maximum cadmium content of 8.26 nmol Cd^{2+}/mg (dry weight) of cells for the resting cell carrying pVINPEC20 was 12-fold higher than that

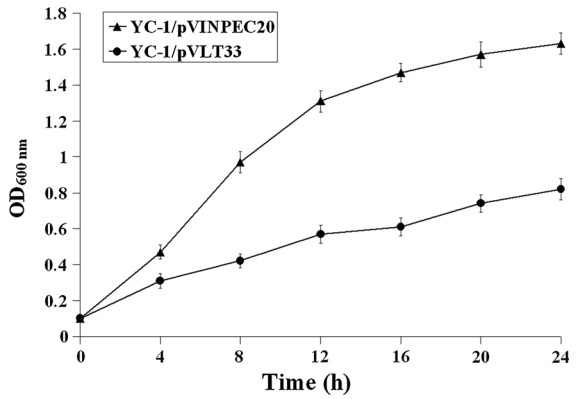

Fig. 2 Growth curves of *Stenotrophomonas* sp. YC-1 harboring pVLT33 or pVINPEC20 in the presence of 80 μM of $CdCl_2$. Cells were grown in LB medium containing 50 μg/ml kanamycin, 1 mM IPTG, and 80 μM of $CdCl_2$. Cells were inoculated at $OD_{600} = 0.1$ into LB medium. Data are mean values ± standard deviations from three replicates

of the control cell carrying pVLT33, suggesting that the enhanced binding effect by the EC20 moiety was sustained even under slow-growing conditions.

More importantly, expression of INPNC-EC20 fusion protein also conferred increased cadmium resistance to the engineered strain. The final cell density for *Stenotrophomonas* sp. YC-1 carrying pVINPEC20 was two-fold higher than the control strain carrying pVLT33 grown in the presence of 80 μM $CdCl_2$ (Fig. 2). Moreover, the control strain carrying pVLT33 grown in the absence of cadmium reached a final cell density similar to that of the recombinant strain carrying pVINPEC20 grown in the presence of cadmium.

Restoration of chlorpyrifos degradation by surface-expressed EC20

To investigate the effect of cadmium on chlorpyrifos degradation, *Stenotrophomonas* sp. YC-1 carrying pVINPEC20 was grown overnight in either the presence or absence of $CdCl_2$. Cells were harvested and resuspended to an OD_{600} of 1.0 in PBS buffer. Resting cells were then incubated with 0.4 mM chlorpyrifos, and the rate of chlorpyrifos degradation was determined in either the presence or absence of $CdCl_2$. In the presence of 16 μM Cd^{2+}, the rate of chlorpyrifos degradation was decreased by 76 % for the engineered strain (Fig. 3). This result confirms that the presence of cadmium is detrimental to chlorpyrifos degradation. In

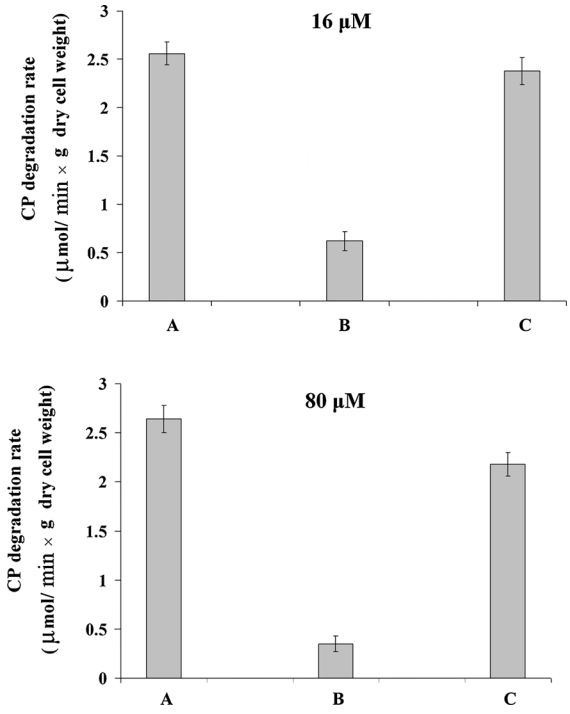

Fig. 3 The chlorpyrifos degradation rate for *Stenotrophomonas* sp. YC-1 harboring pVINPEC20 incubated with either 16 or 80 µM $CdCl_2$. *Stenotrophomonas* sp. YC-1 harboring pVINPEC20 was grown overnight at 200 rpm and 30 °C in LB medium containing 50 µg/ml kanamycin and 0.4 mM chorpyrifos in either the presence or absence of $CdCl_2$ and IPTG. Cells were harvested and resuspended to an OD_{600} of 1.0 in PBS buffer. Resting cells were then incubated with 0.4 mM chlorpyrifos at 200 rpm and 30 °C, and the rate of chlorpyrifos degradation was determined in either the presence or absence of $CdCl_2$. (*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. Data are mean values ± standard deviations from three replicates

contrast, the chlorpyrifos degradation rates were completely restored for the engineered strain expressing EC20 on the surface. A similar protective effect on chlorpyrifos degradation was observed even when the Cd^{2+} concentration was increased by fivefold to 80 µM. These results indicate that the surface-expressed EC20 moiety could effectively sequester Cd^{2+} ions, minimizing transport, and the inhibitory effect of intracellular Cd^{2+} on chlorpyrifos degradation.

Discussion

Bioremediation of soil co-contaminated with heavy metals and organics is considered difficult because the presence of heavy metals is known to inhibit the activity of many organic-degrading microorganisms (Said and Lewis 1991; Sandrin and Maier 2003). Cadmium and chlorpyrifos are often present together in many ecosystems. To date, microorganisms with both cadmium accumulation and chlorpyrifos degradation capabilities have not yet been reported. In this study, a chlorpyrifos-degrading bacterium, *Stenotrophomonas* sp. YC-1, which contains a chromosome-based *mpd* gene encoding MPH responsible for hydrolysis of chlorpyrifos to TCP, was engineered with the capability to sequester heavy metals. The strategy is to display the metal-binding peptide EC20, which has high affinity for a wide range of heavy metals, onto the cell surface in order to eliminate the metal-induced inhibition on chlorpyrifos degradation.

Bacteria such as *E. coli* and *Moraxella* sp. expressing EC20 on the cell surface have been shown to accumulate up to 25-fold more cadmium than the wild-type strain (Bae et al. 2000, 2002). Expression of EC20 on the cell surface of *Stenotrophomonas* sp. YC-1 dramatically increased whole-cell accumulation of Cd^{2+} (12-fold higher than that of the control strain). In this study, the surface expression vector pVINPEC20 allowed the expression of sufficient quantities of INPNC-EC20 fusion protein on the cell surface of *Stenotrophomonas* sp. YC-1, resulting in a recombinant strain with high cadmium affinity. This remarkably high level of surface expression of EC20 could be due to improved membrane translocation of INPNC-EC20 fusion protein in *Stenotrophomonas* sp. YC-1. This can be attributed to the compatibility of the INP anchor with the membrane structure of *Stenotrophomonas* sp. YC-1 since INP was originally isolated from a phylogenetically close species, *Pseudomonas syringae* INA5 (Schmid et al. 1997). Owing to its very high affinity to heavy metals and the high level of expression, cells expressing INPNC-EC20 fusion protein may be very useful as microbial biosorbents for heavy metal removal.

Moreover, surface expression of INPNC-EC20 fusion protein enhanced the tolerance of the engineered strain to cadmium. No cell growth inhibition was observed for the engineered strain expressing INPNC-EC20 fusion protein in the presence of cadmium, and the engineered strain reached a final cell density similar to the control strain carrying pVLT33 after 24 h of incubation. These results suggest

that the presence of the EC20 moiety on the cell surface could effectively sequester extracellular Cd^{2+} ions, alleviate the cellular toxicity of cadmium, and provide a competitive growth advantage to the engineered strain in Cd^{2+}-contaminated environments.

As with most organic degrading microorganisms, the chlorpyrifos degradation rate was significantly impaired (up to 76 %) in the presence of 16 μM Cd^{2+} (Fig. 3). Surface display of EC20 enhanced not only cadmium binding, but also protected the engineered strain against the toxic effects of cadmium on chlorpyrifos degradation. These results suggest that the surface-displayed EC20 was effective in minimizing the inhibitory intracellular Cd^{2+} concentration by extracellular sequestration. Cells with surface-displayed EC20 could maintain the high chlorpyrifos degradation rate in the presence of Cd^{2+} lower than 150 μM, but the rate of chlorpyrifos degradation decreased by 60 % in the presence of Cd^{2+} more than 200 μM, suggesting that the Cd^{2+} absorption capacity of a cell can be saturated. The success of this approach depends on the concentration of Cd^{2+} as well as the concentration of cells.

Members of the genus *Stenotrophomonas* were shown to degrade a wide range of recalcitrant pollutants, and these bacteria are robust and ubiquitous in soil (Liu et al. 2007; Ryan et al. 2009). The host strain *Stenotrophomonas* sp. YC-1 used in this study, isolated from the activated sludge of a wastewater-treatment system of an organophosphate insecticide manufacturer, has been identified as a plant growth-promoting rhizobacteria (PGPR) and successfully used for the remediation of chlorpyrifos-contaminated soil (Yang et al. 2006). Therefore, inoculation of the engineered strain into plant roots should provide a technology useful for the remediation of chlorpyrifos and cadmium co-contaminated soil. This feasibility is currently under investigation.

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