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Cell surface display of synthetic phytochelatins using ice nucleation protein for enhanced heavy metal bioaccumulation

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Abstract

Synthetic phytochelatins (ECs) composed of $(Glu–Cys)_nGly$ are protein analogs of phytochelatin that exhibit improved metal-binding capacity over metallothioneins (MTs). Expression of EC20 on the surface of *E. coli* using the Lpp-OmpA anchor resulted in improved bioaccumulation of cadmium and mercury, providing a new method for treating heavy metal contamination. To further improve the whole-cell accumulation of heavy metals, EC20 was expressed on the surface of *Moraxella* sp., a bacterium known to survive in contaminated environments, using the truncated ice nucleation protein (INPNC) anchor. Production of EC20 was approximately three-fold higher in *Moraxella* sp. than *E. coli*. As a consequence, the mercury-binding capacity of the recombinant *Moraxella* sp. was increased by more than 10-fold. Owing to the very high level of surface expression, the use of *Moraxella* sp. and INPNC anchor may prove to be useful for the remediation of other environmental contaminants.

Keywords: Mercury; Cadmium; Heavy metal removal; Decontamination

1. Introduction

Because of their intrinsically persistent nature, heavy metal ions (As, Cd, Cr, Cu, Hg, Ni, Pb, Se, V and Zn) are major contributors to pollution of the biosphere [1]. These metals when discharged or transported into the environment may undergo transformations and can have a large environmental, public health, and economic impact [2]. The increasingly restrictive Federal regulation of allowable levels of heavy metal discharge and accelerated requirements for the remediation of contaminated sites make necessary the development of new approaches and technologies for heavy metal removal. Conventional technologies such as precipitation–filtration, ion-exchange, reverse osmosis, oxidation–reduction, and membrane separation are often inadequate to reduce metal concentration to acceptable regulatory standards [3]. Recent research has focused on the development of novel bioadsorbents with increased affinity, capacity, and selectivity for target metals.

Immobilization is the major mechanism employed by nature (animals and plants) for counteracting heavy metal toxicity [4]. Metallothioneins (MTs) and phytochelatins (PCs) are two groups of naturally occurring, cysteine-rich peptides that are synthesized for binding a wide range of heavy metals [5]. Expression of MTs in *E. coli* to improve the bioadsorption of heavy metals is a promising technology for the development of microbial-based biosorbents [6]. However, metal removal by intracellular MTs has been problematic because of the limited metal uptake [7]. One approach to circumvent this problem is to directly anchor the MTs onto the cell surface [8]. Suggestions have been made to use PCs in a similar manner as MTs since PCs offer many advantages, particularly the higher metal-binding capacity on a per cysteine basis [9]. Because of the presence of a γ bond between amino acids, the exact biochemical and genetic mechanisms for their synthesis and chain elongation have not been elucidated. However, protein analogs of PC with the general structure $(Glu–Cys)_nGly$ (defined as synthetic phytochelatins or ECs) have been shown to bind heavy metals with similar efficiencies as PCs [10]. We recently demonstrated that *E. coli* strains with EC20 (20 cysteines) displayed on the cell surface using the Lpp-OmpA fusion system accumulated a substantially higher amount of cadmium than the wild-type cells [11].

However, lab-born *E. coli* strains are not suitable for in situ soil remediation, since they are not adapted to these

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environments. A more realistic approach is to engineer soil bacteria that are known to survive in contaminated environments for an extended period. Particularly interesting are *Pseudomonas* and related species such as *Moraxella* sp. [12]. The initial challenge is to develop an efficient system to target EC20 on the surface of these gram-negative bacteria. We recently demonstrated that expression of organophosphorus hydrolase on the cell surface was more efficient in *Moraxella* sp. than in *E. coli* using the ice nucleation protein anchor [13]. This strategy could be used to further improve the whole-cell accumulation of heavy metal with surface-expressed EC20. In this paper, we demonstrated the surface-expression of ECs in *Moraxella* sp. using the ice nucleation protein anchor. The resulting recombinant strains accumulated more than 10-fold higher mercury than the wild-type *Moraxella* sp..

2. Materials and methods

2.1. Strains, plasmids, media, and general procedures

E. coli strain JM109 (*endA*1, *thi, rps*L, *sbcB*15, *hsdR*4, Δ(*lac-proAB*), [F′, *traD*36, *proAB*, *lacI*^{q}ZΔM15]) and a *Moraxella* sp. [12] isolated from activated sludge by selective enrichment with PNP [12] were used in this study. Plasmids pLO20 [11] and pINP20 were used to express the Lpp-OmpA-EC20 and INPNC-EC20 fusions in *E. coli*, respectively. Plasmid pPNC20, a derivative of pVLT33 [19] carrying the *inpnc-ec20* gene was used for surface expression in *Moraxella* sp.

Cultures were grown in either MJS medium or LB medium supplemented with either 50 μg/ml ampicillin or kanamyacin at 30 °C to an OD_{600} of 0.3 when 1 mM IPTG was added to induce the expression of the fusion proteins.

2.2. Construction of ice-nucleation fusions

The INPNC-EC20 fusion was constructed as follows. The *ec20* fragments were PCR amplified from plasmid pVT20 using the primers: ec-c) 5′ GCT*GGATCC*TATGGAATGTG 3′ and ec-d) 5′ GCAAGGTAGACAAGCCG 3′. The amplified fragment was digested with *Bam*HI and *Hind*III, gel-purified and subcloned into a similarly digested pINCOP [15] resulting in pINP20. The resulting construct allows expression of EC20 on the surface of *E. coli*. For surface expression in *Moraxella* sp., the entire *inpnc-ec20* fragment was excised with *Eco*RI/*Hind*III and ligated into pVLT33 to create pPNC20.

2.3. Western blot analysis

Samples (10 μl) of concentrated cells at OD=10 were mixed with loading buffer and boiled for 10 min. Samples were run on a 10% (w/v) acrylamide sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel. Proteins were then transferred to a nitrocellulose support before incubation with INPNC-specific antibodies. Western blot analysis was performed using a Bio-Rad Immun-Blot GAR-AP kit (BioRad, Hercules, CA). Prestained broad-range molecular weight markers were used to estimate protein weights. The intensity of the bands was quantified using a BioRad Gel Doc 2000 Gel Documentation System and the Quantity One software.

2.4. Immunofluorescence microscopy

Following 2 days incubation, cells were harvested and resuspended (0.5 OD_{600}) in phosphate-buffered saline (PBS) buffer with 3% bovine serum albumin (BSA). Intact cells were then incubated with rabbit anti-INPNC antisera (1:3000) [14] for 8 h at 4 °C. The cells were washed extensively, resuspended in PBS with secondary antibody goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC; Sigma) at a dilution 1:64 and incubated overnight at 4 °C. Prior to microscopy, cells were washed five times with PBS. Photographs were taken using an immunofluorescence microscope (Nikon).

2.5. Bioaccumulation of Cd^{2+}

Cells were grown in MJS medium and induced with 1 mM IPTG for the expression of fusion proteins. $CdSO_4$ (100 μM) was added to the culture in order to allow expression of ECs in the presence of Cd^{2+} in order to prevent oxidation [8]. Cells were harvested after 20 h induction, washed twice with double-distilled water, and treated overnight with concentrated nitric acid. Disrupted cells were then diluted with double-distilled water and centrifuged for 10 min at 4 °C. The concentration of metals in the soluble fraction was directly measured through an atomic absorption spectrophotometer (Perkin Elmer AAS3100).

2.6. Bioaccumulation of Hg^{2+}

Cells were incubated overnight in LB media with either ampicillin (100 μg/ml) or kanamycin (50 μg/ml) at 30 °C. Overnight cultures were diluted to an OD_{600}=0.1 in fresh LB media containing appropriate antibiotics. After 2 h, 1 mM IPTG and 5 μM $HgCl_2$ were added. After 7 h, aliquots of cultures were taken to determine the amount of accumulated mercuric ions and the expression of desired fusion proteins. For mercury analysis, cells were washed twice with distilled water and digested with concentrated nitric acid. Total mercury was analyzed by cold vapor atomic absorption spectrophotometry using a mercury analyzer (Coleman Model 50B).

3. Results and discussion

3.1. Bioaccumulation of heavy metals by recombinant E. coli expressing INPNC-EC20

Ice-nucleation protein (INP) is an outer membrane protein from *Pseudomonas syringae* that acts as a template for ice nucleation [15]. Both INP and the truncated version of INP containing only the N- and C-terminal portion (INPNC) can be used to target proteins to the cell surface of *E. coli* [16], *Salmonella* [17], and *Morxella* sp. [13]. The InaV protein from *Pseudomonas syringae* INA5 was chosen because it has been shown to target proteins on the cell surface more efficiently than the previously reported InaK anchor [13]. Plasmid pINP20, carrying the *inpnc-ec20* fusion was constructed by inserting the *ec20* fragment into the vector pINCOP [16]. Expression of INPNC-EC20 was confirmed by blotting with INPNC antisera. A band of approximately 35 kDa was detected from cells carrying pINP20 (Fig. 1), while no such protein was detected with JM109 cells carrying pUC18.

One of the problems associated with surface expression using the Lpp-OmpA fusion system is the severe growth inhibition [18]. In the case of cultures carrying pLO20, cell growth was virtually stopped after induction. On the other hand, cells carrying pINP20 showed no sign of growth inhibition even after induction (Fig. 2). The final cell density was more than two-fold higher than cells carrying pLO20. However, whole-cell accumulation of Cd^{2+} was only 50% that of cells carrying pLO20. This difference is consistent with a lower level of EC20 expressed on the cell surface using the INPNC anchor (data not shown).

To demonstrate the binding capability of EC20 to other heavy metals, Hg^{2+} accumulation of the different *E. coli* strains was investigated. As shown in Fig. 3, *E. coli* strain carrying pUC18 accumulated a very low level of Hg^{2+} and whole-cell accumulation of Hg^{2+} was increased with EC20 expressed on the surface. Again, the level of accumulation was three-fold higher with the Lpp-OmpA anchor. We have recently demonstrated that surface expression of proteins is more efficient in *Moraxella* sp. than in *E. coli* using the ice nucleation protein anchor [13], an alternative strategy to further improve the whole-cell accumulation of heavy metal may be to express EC20 on the surface of *Moraxella* sp..

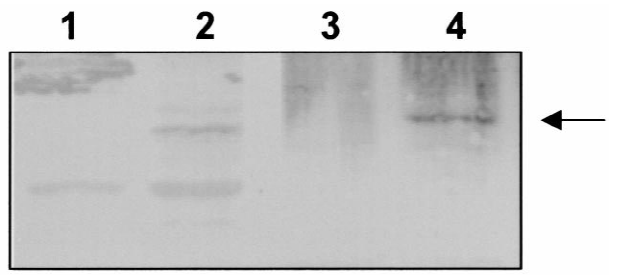

Fig. 1. Western blot analysis of INPNC-EC20 expression. Total protein extracts (10 μl) from *E. coli* carrying pUC18 (lane 1) or pINP20 (lane 2) and *Moraxella* sp. carrying pVLT33 (lane 3) or pINP20 (lane 4) were blotted with the INPNC antisera.

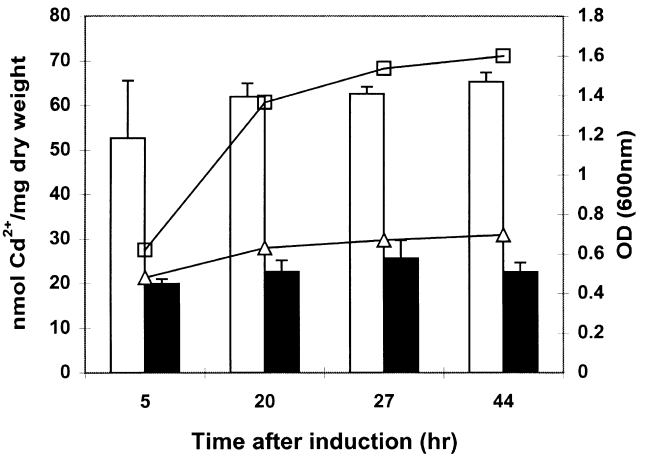

Fig. 2. Cell growth (△, pLO20 and □, pINP20) and Cd^{2+} accumulation (white bar, pLO20 and black bar, pINP20) for JM109 cells expressing EC20 on the surface using either the Lpp-OmpA (pLO20) or INPNC (pINP20) anchor.

3.2. Bioaccumulation of mercury by recombinant Moraxella sp. expressing INPNC-EC20

For expression of INPNC-EC20 in *Moraxella* sp., plasmid pPNC20, carrying the *inpnc-ec20* fusion was constructed by inserting the *inpnc-ec20* fragment into a shuttle vector pVLT33 [19]. Expression of the INPNC-EC20 fusion was under control of a *tac* promoter. Induced cultures of *Moraxella* sp. were viable during prolonged incubation for 24 h. Although EC20 was expressed in both *E. coli* and *Moraxella* sp. as demonstrated by Western blotting with the INPNC antiserum, the level of expression was about three-fold higher in *Moraxella* sp. as judging from the intensity of the band corresponding to INPNC-EC20 (Fig. 1). To investigate whether the INPNC-EC20

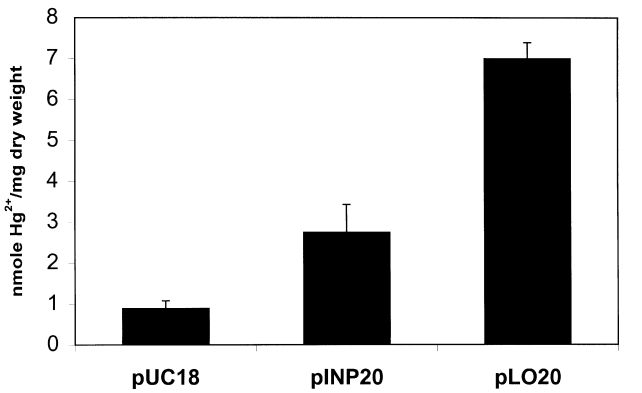

Fig. 3. Bioaccumulation of Hg^{2+} by *E. coli* strains expressing EC20 on the cell surface using either the Lpp-OmpA (pLO20) or INPNC (pINP20) anchor. Cells carrying pUC18 were used as the control. Data were obtained from two independent experiments.

fusion proteins were displayed on the bacterial 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 FITC-labeled goat anti-rabbit IgG antibody. As shown in Fig. 4A, cells harboring pPNC20 were brightly fluorescent, indicating that the INPNC fusions was successfully displayed on the surface. Cells carrying only pVLT33 were not stained at all with the FITC-labeled secondary antibody (Fig. 4B). A similar result was also observed with the different *E. coli* strains.

The Hg^{2+} bioaccumulation capability of whole cells expressing EC20 was tested by monitoring the binding of Hg^{2+} by cold-vapor atomic absorption spectroscopy (Coleman Model 5B Mercury Analyzer System). As shown in Fig. 5, strains expressing EC20 on the cell surface accumulated a significantly higher amount of Hg^{2+} than cells carrying pVLT33. The amount of Hg^{2+} accumulated was almost 10-fold and three-fold higher than that of *E. coli* expressing INPNC-EC20 or Lpp-OmpA-EC20, respectively. This result is consistent with the higher level of surface-expressed EC20 in *Moraxella* sp..

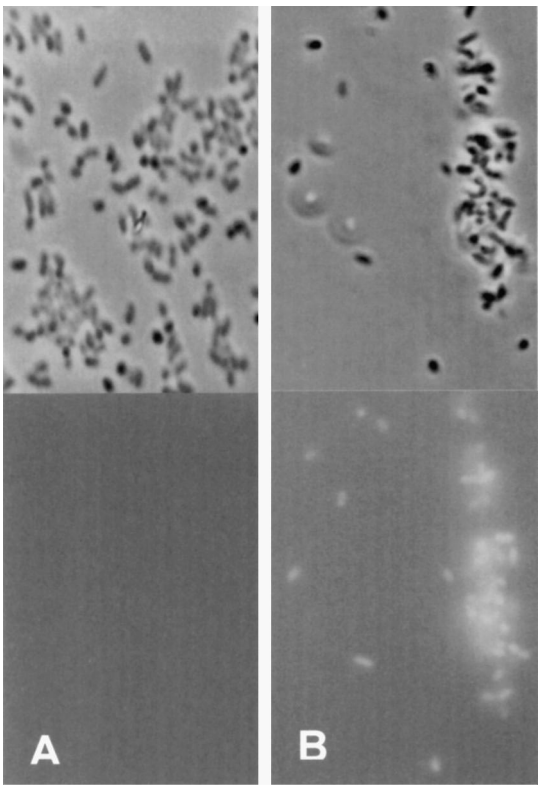

Fig. 4. Immunofluorescence micrographs of *Moraxella* sp. harboring (A) pVLT33 and (B) pPNC20. Cells were probed with anti-INPNC antiserum and fluorescently stained with goat anti-rabbit IgG–FITC conjugate. Pictures of the same cells obtained in the absence of UV excitation were shown on top.

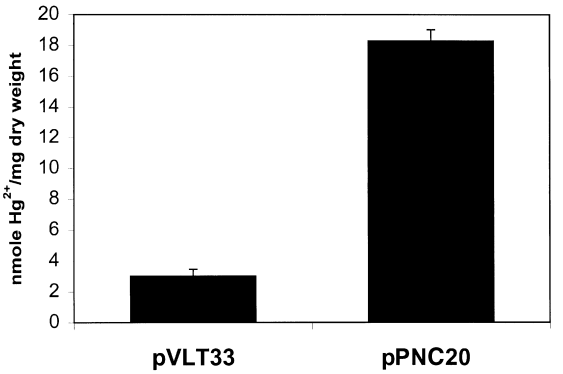

Fig. 5. Bioaccumulation of Hg^{2+} by *Moraxella* sp. expressing INPNC-EC20 (pPNC20) on the cell surface. Cells carrying pVTL33 were used as the control. Data were obtained from two independent experiments.

4. Conclusions

A genetically engineered *Moraxella* sp. was developed for enhanced bioaccumulation of heavy metals. The INPNC anchor was used to anchor EC20 on the surface of the cells, providing a 10-fold increase in their mercury binding capacity. Since the expression of surface protein is more efficient in *Moraxella* sp. than *E. coli* using the INPNC anchor, this robust bacterium may be an ideal host for the remediation of other contaminants using this surface display strategy.

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