Enhanced Bioaccumulation of Heavy Metals by Bacterial Cells Displaying Synthetic Phytochelatins

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Abstract: A novel strategy using synthetic phytochelatins is described for the purpose of developing microbial agents for enhanced bioaccumulation of toxic metals. Synthetic genes encoding for several metal-chelating phytochelatin analogs $(Glu\text{-}Cys)_nGly$ (EC8 (n = 8), EC11 (n = 11), and EC20 (n = 20)) were synthesized, linked to a *lpp-ompA* fusion gene, and displayed on the surface of *E. coli*. For comparison, EC20 was also expressed periplasmically as a fusion with the maltose-binding protein (MBP-EC20). Purified MBP-EC20 was shown to accumulate more Cd^{2+} per peptide than typical mammalian metallothioneins with a stoichiometry of 10 Cd^{2+}/peptide. Cells displaying synthetic phytochelatins exhibited chain-length dependent increase in metal accumulation. For example, 18 nmoles of Cd^{2+}/mg dry cells were accumulated by cells displaying EC8, whereas cells exhibiting EC20 accumulated a maximum of 60 nmoles of Cd^{2+}/mg dry cells. Moreover, cells with surface-expressed EC20 accumulated twice the amount of Cd^{2+} as cells expressing EC20 periplasmically. The ability to genetically engineer ECs with precisely defined chain length could provide an attractive strategy for developing high-affinity bioadsorbents suitable for heavy metal removal. © 2000 John Wiley & Sons, Inc. *Biotechnol Bioeng* **70**: 518–524, 2000.

Keywords: heavy metals; bioadsorbents; Lpp-OmpA; phytochelatin analogs.

INTRODUCTION

The discharge of heavy metals due to agricultural, industrial, and military operations has serious adverse effects on the environment (Ji and Silver, 1995; Nriagu and Pacyna, 1989). Higher organisms respond to the presence of heavy metals with the production of cysteine-rich peptides such as glutathione (GSH) (Singhal et al., 1997), phytochelatins (PCs), and metallothioneins (MTs) (Mehra and Winge, 1991) that bind metal ions (such as cadmium, lead, mercury, copper) and sequester them in biologically inactive forms (Hamer, 1986; Stillman et al., 1992). Overexpression of MTs in bacterial cells results in enhanced metal accumulation and thus offers a promising strategy for the development of microbial-based biosorbents to remediate metal contamination (Kille et al., 1991; Pazirandeh et al., 1995; Romeyer et al., 1990). However, metal removal by intracellular MTs has been problematic because of the limited metal uptake (Chen and Wilson, 1997) and the inability to recycle intracellularly accumulated metals (Gadd and White, 1993). One clever solution to bypass this problem is to express MTs on the cell surface. Sousa et al. (1996) demonstrated this possibility by inserting MTs into the permissive site 153 of the LamB sequence. Expression of the hybrid proteins on the cell surface dramatically increases whole-cell accumulation of Cd^{2+}.

PCs are short, cysteine-rich peptides with the general structure $(\gamma Glu\text{-}Cys)_nGly$ (n = 2–11) (Rauser, 1995; Zenk, 1996). PCs offer many advantages over MTs due to their unique structural characteristics, particularly the continuously repeating γGlu-Cys units. For example, PCs have higher metal-binding capacity (on a per cysteine basis) than MTs (Mehra and Mulchandani, 1995). In addition, PCs can incorporate high levels of inorganic sulfide that results in tremendous increases in the Cd^{2+}-binding capacity of these peptides (Mehra et al., 1994). Suggestions have been made to use PCs in a similar manner as MTs (Zenk, 1996). However, development of organisms overexpressing PCs requires a thorough knowledge of the mechanisms involved in the synthesis and chain elongation of these peptides. The presence of a γ bond between glutamic acid and cysteine in PCs indicates that these peptides must be synthesized enzymatically. PC biosynthesis may proceed by a variety of reactions involving enzymes that transfer γGlu-Cys from GSH to GSH or other PCs (Zenk, 1996). Although PC synthase has now been cloned (Clements et al., 1999; Ha et al., 1999; Vatamaniuk et al., 1999), factors that govern chain elongation of PCs are far from understood.

An attractive alternative strategy is to develop organisms harboring synthetic genes encoding protein analogs of PC with the general structure $(Glu\text{-}Cys)_nGly$ (ECs). These peptides differ from PCs because the peptide bond between

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glutamic acid and cysteine is the standard α peptide bond that can be synthesized on the ribosomal machinery (Fig. 1). Because of the difference in γ and α bonding, the metal-binding affinity between ECs and PCs may be dramatically different. However, detailed experiments with EC2 and EC4 have shown that these peptides bind a variety of metals in a manner similar to that exhibited by PC2 and PC4 (Bae and Mehra, 1997; Dameron and Winge, 1990). Although the metal-binding stoichiometries for ECs with higher cysteine content are still to be established, it is easy to envision that they might work in a similar fashion as EC2 and EC4. More importantly, it is possible to produce large quantities of ECs with any defined chain length of interest.

Recently, a gene fusion system consisting of the signal sequence and the first nine amino acids of lipoprotein (Lpp) joined to a transmembrane domain from outer membrane protein A (OmpA) has been used successfully to anchor a variety of proteins and enzymes onto the cell surface (Francisco et al., 1992, 1993; Richins et al., 1997). In this article, we describe the construction and characterization of recombinant *E. coli* strains that anchor and display functional synthetic phytochelatins ranging from 8–20 cysteines (EC8, EC11, and EC20) onto the cell surface using this Lpp-OmpA fusion system. We demonstrate that these synthetic phytochelatins confer metal-binding capability on the host cells and the resulting novel bioadsorbents accumulate a substantially higher amount of cadmium than the wild-type cells.

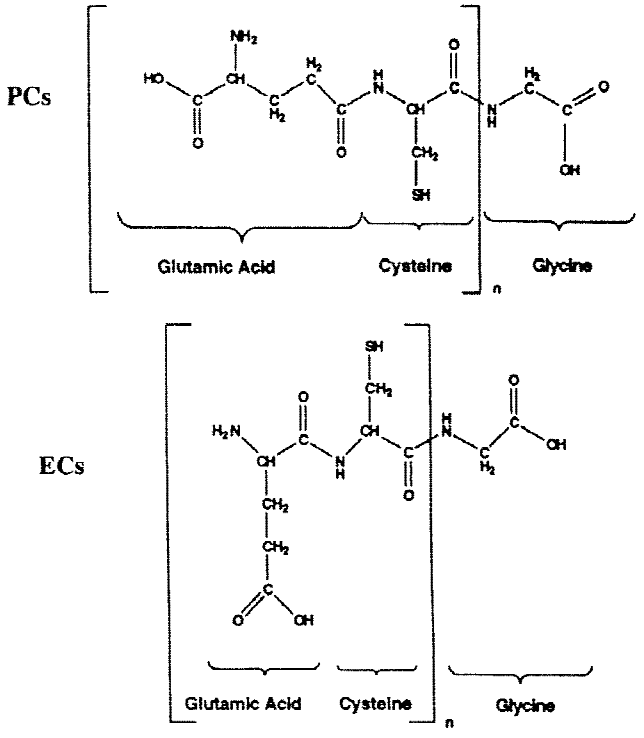

Figure 1. Chemical structures of natural (PCs) and synthetic (ECs) phytochelatins. Note the different in linkages between glutamic acid and cysteine.

MATERIALS AND METHODS

Strains, Plasmids, Media, and General Procedures

E. coli strain JM105 (*endA*1, *thi*, *rps*L, *sbcB*15, *hsdR*4, Δ(*lac-proAB*), [F′, *traD*36, *proAB*, *lacI*^{q} ZΔM15]) was used as the recipient of all plasmids. Plasmids pLO8, pLO11, and pLO20 are derivatives of the vector pUC18, which bears the sequence coding for Lpp-OmpA-EC8, Lpp-OmpA-EC11, and Lpp-OmpA-EC20, respectively. pM20 is a derivative of pMAL-p2x (New England BioLabs, Beverly, MA), which allows the expression of EC20 in the periplasm. Cultures were grown in low-phosphate MJS medium (Sambook et al., 1989) supplemented with 50 μg/ml ampicillin at 30°C to an OD_{600} of 0.3 when 1 mM IPTG was added to induce the expression of the fusion proteins. 100 μM $CdSO_4$ was subsequently added for metal binding experiments. General molecular biology procedures followed standard protocols unless specified otherwise (Sambook et al., 1989).

Construction of Lpp-OmpA-EC20 Fusion Protein

The synthetic gene encoding for $(Glu\text{-}Cys)_{20}Gly$ (EC20) was prepared using two oligonucleotides (Research Genetics, Huntsville, AL): ec-a) 5′TTT*GGATCC*ATGGAATGTGAATGTGAATGTGAATGTGAATGTGAATGTGAATGTGAGTGTGAATGTGAGTGCGAATGCGAA3′ and ec-b) 5′TTT*AAGCTT*TTAACCACATTCACATTCACATTCACATTCACATTCACATTCGCATTCACATTCGCATTCGCATTCGCACTC3′. The two oligonucleotides were mixed, boiled, and cooled to hybridize at the underlined sequence. The preferred codons for glutamic acid (GAA) and cysteine (TGT) have been changed at some locations to less frequently used codons, GAG and TGC, respectively, to prevent unwanted hybridization. Double-strand synthesis was accomplished using the Klenow fragment (Promega, Madison, WI). The synthetic gene was digested with *Bam*HI (italicized) and *Hind*III (italicized) and the resulting fragment was cloned into the yeast-*E. coli* shuttle vector pVT102-U (Vernet et al., 1987), digested with the same restriction enzymes to generate pVT20. The resulting clone was sequenced to confirm the presence of the correct *ec20* fragment. Construction of synthetic genes for EC8 and EC11 followed similar procedures.

To construct the *lpp-ompA-ec20* fusion, the *lpp-ompA* fragment (481 bp) was PCR amplified as described previously (Richins et al., 1997), digested with *Eco*RI and *Kpn*I and cloned into pUC18 to generate plasmid pLO. The *ec20* fragments were PCR amplified from plasmid pVT20 using the primers: ec-c) 5′ GCT*GGATCC***T**ATGGAATGTG 3′ and ec-d) 5′ GCAAGGTAGACAAGCCG 3′. The primer ec-c contains an extra base (bold) just behind the *Bam*HI site in order to generate an in-frame fusion with *lpp-ompA*. The amplified fragment was digested with *Bam*HI and *Hind*III, gel-purified, and subcloned into pLO to generate pLO20. The cloning of the *ec20* fragment was again con-

firmed by DNA sequencing. The recombinant plasmid pLO20, coding for Lpp-OmpA-EC20, was used for all subsequent experiments. Plasmids pLO8 and pLO11 containing the genes encoding Lpp-OmpA-EC8 and Lpp-OmpA-EC11 were generated similarly.

To express EC20 in the periplasm, the *Bam*HI and *Hind*III digested fragment of *ec20* was cloned into pMAL-p2x, which allows the expression of EC20 as a fusion to the maltose-binding protein. Purification of the MBP-EC20 fusions was achieved using an amylose affinity column as recommended by the manufacturer (New England Biolabs). Fractionation of total proteins into cytoplasmic and periplasmic fractions was achieved by the cold osmotic shock procedure (Neu and Heppel, 1965).

Radiolabeling the Target Proteins and SDS-PAGE Analysis

Radiolabeled cysteine (^{35}S, 1,075 Ci/mmol, ICN) was added at the time of induction (final concentration of 5 μCi/ml). After the desired time of induction, 1.5 ml aliquot of each culture was centrifuged. The extracted total proteins were boiled in sample buffer (Sambook et al., 1989) for 5 min and separated by SDS-PAGE (12.5% (w/v) polyacrylamide) (Laemmli, 1970). The gel was dried and exposed to X-ray film.

Protease Accessibility Experiments

JM105 cells transformed with pLO20 were grown in MJS media. Appropriate amounts of IPTG (1 mM), Cd^{2+} (0.1 mM), and [^{35}S]-cysteine (5 μCi/ml) were added to the culture at $OD_{600} = 0.3$. 1.5 mL of the culture was centrifuged after the desired time of induction. The cell pellets were resuspended in the incubation buffer (15% (w/v) sucrose, 15 mM Tris-HCl, pH 7.8) to adjust $OD_{600} = 10$. Proteinase K (Sigma, St. Louis, MO) was added to the resuspended cells at a final concentration 10 μg/ml. 10 μM PMSF was added to inactivate proteinase K after the incubation. Following incubation up to 21 h at room temperature, 100 μl of sample was mixed with 20 μl of 6X sample buffer. The extracted total proteins were boiled for 5 min and separated by SDS-PAGE (12.5% (w/v) polyacrylamide) (Laemmli, 1970). The gel was dried and exposed to X-ray film. JM105 cells transformed with pM20 was used as the control.

Cd^{2+} Binding with Purified MBP-EC20

MBP-EC20 was purified through amylose column as recommended by the manufacturer (New England Biolabs). The purity of the protein was confirmed through SDS-PAGE (12.5% (w/v) polyacrylamide) (Laemmli, 1970). 10 nmoles of the purified fusion protein was incubated in 50 mM Tris-Cl buffer (pH 7.4) supplemented with 5 mM DTT for 2 h at 37°C to reduce the thiol groups. 300 nmoles of Cd^{2+} was subsequently added to the solution and incubated for 1 h at 37°C. The sample was fractionated through a Sephadex G-25 column to separate the Cd^{2+}-protein complex from free Cd^{2+} and DTT. The concentrations of Cd^{2+} and protein in each fraction were analyzed by flame atomic absorption spectrophotometry (Perkin Elmer, Norwalk, CT; AAS3100) and thiol analysis (Grassetti and Murray, 1967), respectively.

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+}. Cells did not show any significant reduction in growth at this concentration of the metal. Cells were harvested after the desired time of 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 Cd^{2+} in the soluble fraction was directly measured through atomic absorption spectrophotometer (Perkin Elmer AAS3100).

RESULTS

Expression of Synthetic Phytochelatins on the Cell Surface

Synthetic genes coding for several synthetic phytochelatin (EC8, EC11, and EC20) were synthesized, linked to *lpp-ompA* fusion gene and displayed on the surface of *E. coli*. For comparison, EC20 was also expressed periplasmically as a fusion with the maltose-binding protein (MBP-EC20). The ability to genetically engineer ECs with precisely defined chain lengths enables us to demonstrate for the first time the metal-binding capability of any PC or EC containing up to 20 cysteines.

The high cysteine content of the synthetic phytochelatins, when labeled with ^{35}S cysteine, enables their ready detection by autoradiography. Although a pUC18-based plasmid was used for expression, the production of Lpp-OmpA-ECs was not detected in uninduced cultures (Fig. 2). A significantly smaller amount of plasmid DNA was recovered from these cultures, indicating a dramatic reduction in the plasmid copy number. This may be a possible defense mechanism preventing excessive expression of a surface protein. However, in the presence of 1 mM IPTG the synthesis of full-size Lpp-OmpA-EC8 (18.5 kDa), Lpp-OmpA-EC11 (19 kDa), and Lpp-OmpA-EC20 (21 kDa) was detected (Fig. 2). The expression level of MBP-EC20 fusions (47 kDa) was more than 10 times higher than expression on the cell surface, an observation consistent with the surface-expression of other Lpp-OmpA fusions (Francisco et al., 1992; Richins et al., 1997). No significant difference in the level of MBP-EC20 fusions was detected between the whole cells and the periplasmic fraction (data not shown),

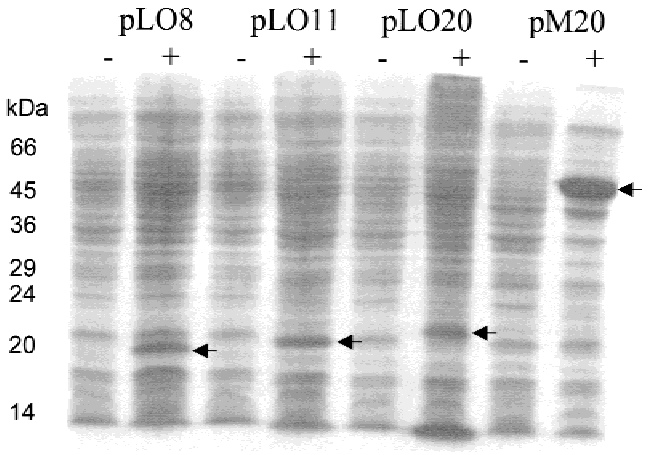

Figure 2. Expression of EC fusion proteins. [^{35}S]cysteine was added to the cultures at OD_{600} = 0.3. The cultures were further grown for 24 h. Total cell proteins were separated on SDS-PAGE (12.5% (w/v) polyacrylamide). The gel was dried and autoradiographed. Expression from uninduced (−) and induced (+) cultures harboring pLO8, pLO11, pLO20, and pM20, respectively, are shown. The molecular weight markers are shown in the far left lane. The desired fusion proteins are marked with arrows.

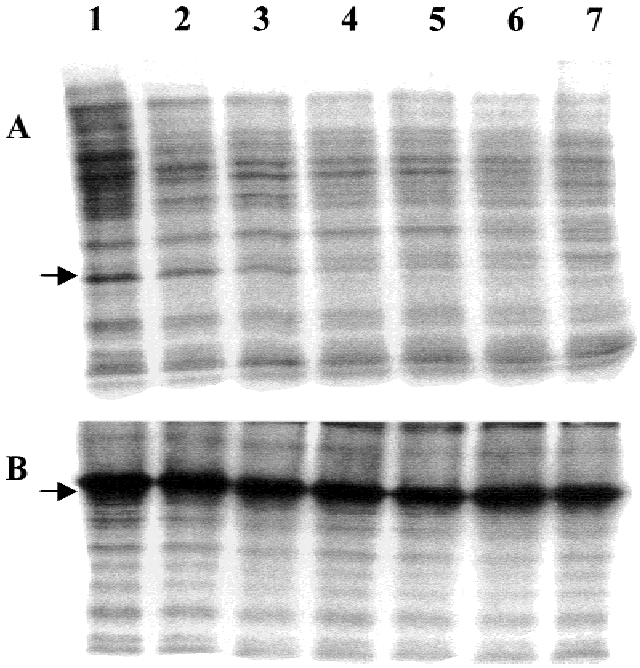

Figure 3. Protease accessibility experiments. Autoradiogram of radiolabeled proteins from *E. coli* cells harboring pLO20 (**a**) and pM20 (**b**) after proteinase K treatment. Lane 1 represents total proteins from *E. coli* cells without proteinase K treatment. Lanes 2–7 represent samples from *E. coli* cells treated with proteinase K for 3 min, 10 min, 1 h, 2 h, 5 h, and 21 h, respectively. The locations of EC20 fusion proteins are indicated with arrows.

confirming the localization of the MBP-EC20 fusion in the periplasm.

The localization of the Lpp-OmpA-EC fusions was confirmed by analyzing ^{35}S-labeled proteins from the soluble and membrane fractions. The majority of the fusion proteins were associated with the cell membrane (data not shown). Protease accessibility experiments were also carried out to ascertain the presence of ECs on the surface. Cultures grown on ^{35}S-labeled cysteine were incubated with and without proteinase K for up to 21 h and the total protein was analyzed by SDS-PAGE. For the cells incubated with proteinase K, the intensity of Lpp-OmpA-EC20 fusions continued to decrease and was no longer detectable after 2 h (Fig. 3). In contrast, no observable decline in the intensity was detected from cells overexpressing the MBP-EC20 fusions even after 21 h of incubation (Fig. 3). These results are consistent with the fact that proteinase K is accessible only to proteins exposed on the cell surface, but not to proteins in the periplasm because proteinase K cannot get across the outer membrane.

Cd^{2+} Binding to EC20

To test the metal-binding capability of synthetic phytochelatins, MBP-EC20 fusion proteins were purified from cultures of JM105 (pM20) grown in the presence of Cd^{2+} using an amylose resin affinity column. The purity of the fusion protein was confirmed through SDS-PAGE and less than one equivalent of Cd^{2+} was found to associate with the purified MBP-EC20 fusions. A significantly higher stoichiometric ratio was obtained for the MBP-EC20 fusions when the proteins were reconstituted with Cd^{2+} after treatment with DTT. The ratios of Cd^{2+} to MBP-EC20 were determined to be 9.9, 10.1, and 9.8 in fractions 6, 7, and 8, respectively (Fig. 4). The concentration of MBP-EC20 was determined by both the thiol assay (Grassetti and Murray, 1967) and the Bradford method (Sambook et al., 1989) to ensure that the thiols in fractions 6–8 are not from the added DTT. Free DTT and DTT-Cd^{2+} complex were eluted in fractions 12–15. Since there is no cysteine residue in MBP, this result reflected the Cd^{2+} binding stoichiometry to EC20.

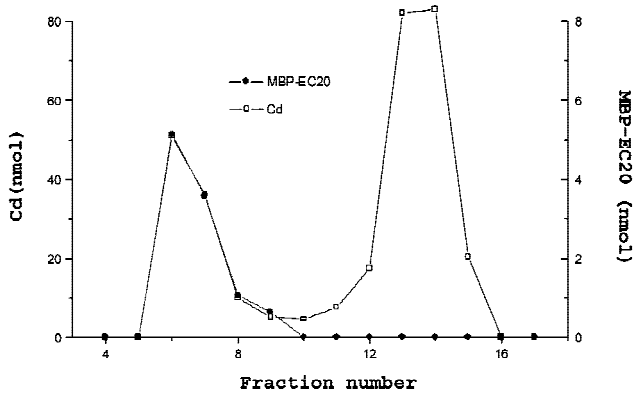

Figure 4. Cadmium binding experiment with purified MBP-EC20 fusions. Ten nmoles of purified fusion protein was first treated with 5 mM DTT and then incubated in 300 nmoles of Cd^{2+}. The resulting mixture was fractionated through a Sephadex G-25 column. The concentrations of Cd^{2+} and protein of each fraction were analyzed by flame atomic absorption spectrophotometry (Perkin Elmer AAS3100) and thiol analysis, respectively.

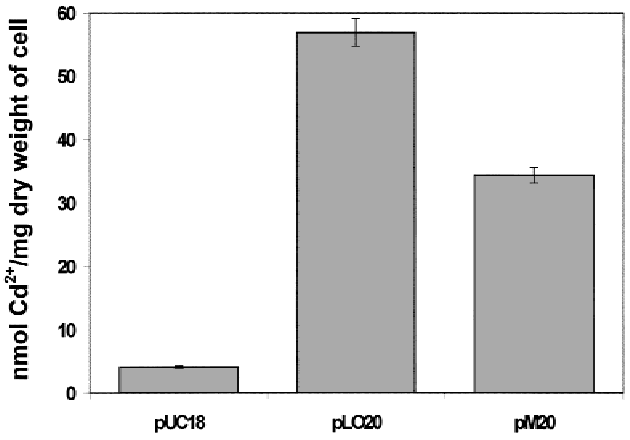

Figure 5. Bioaccumulation of Cd^{2+} by cells expressing fusion EC20 protein. Plasmids pUC18 were used as negative controls. The data were obtained from five independent experiments.

It is generally accepted that mammalian MTs have a stoichiometric ratio of 7 for Cd^{2+} and Zn^{2+} and 12 for copper (Hamer, 1986; Stillman et al., 1992); our results demonstrated that EC20 has 40% higher Cd^{2+} binding capacity than that of MTs.

Bioaccumulation of Cd^{2+} by Surface-Expression of ECs

The metal-binding ability of whole cells expressing EC20 was tested by monitoring the binding of Cd^{2+} to *E. coli* expressing EC20 either on the cell surface (pLO20) or in the periplasm (pM20) through atomic absorption spectrometry. Cells carrying pUC18 were used as the control. Cells were grown in MJS medium supplemented with 0.1 mM Cd^{2+} and metal binding was monitored 16 h after induction. As shown in Figure 5, strains producing EC20 accumulated a substantially higher amount of Cd^{2+} than cells carrying pUC18. However, the benefit of expressing EC20 on the cell surface is more significant, as the amount of Cd^{2+} accumulated by cells with EC20 anchored on the surface was almost twice the amount in cells with EC20 expressed in the periplasm. This is somewhat unexpected since the periplasmic expression of EC20 is much higher than that on the cell surface (Fig. 2). However, analysis of metal binding from MBP-EC20 fusions purified from the culture of JM105 (pM20) revealed that less than one Cd^{2+} was associated with each EC20, a value dramatically lower than the maximum of 10. This phenomenon may be attributed to the limitation in Cd^{2+} uptake resulting in most of the cysteine binding sites being either vacant or oxidized. A similar limitation in mercury transport has recently been demonstrated (Chen and Wilson, 1997). The coexpression of mercury transport proteins greatly improved mercury accumulation by cells overexpressing MTs intracellularly.

The chain length of PCs has been shown to influence metal binding affinity and stoichiometry (Mehra et al., 1996a,b). In the case of ECs, the amount of Cd^{2+} accumulated increased with increasing cysteine residues in the ECs (Fig. 6). Cells with EC20 expressed on the surface (ca. 60 nmol Cd^{2+}/mg dry weight of cell) accumulated almost twice the amount of Cd^{2+} as compared to cells expressing EC11. This result is consistent with the increasing number of metal-binding centers present.

DISCUSSION

The de novo design of metal-binding peptides is an attractive alternative to MTs, as they offer the potential of enhanced affinity and selectivity for heavy metals. Peptides with an abundance of cysteine residues, for example, are known to bind Cd^{2+} and Hg^{2+} with very high affinity. PCs are naturally occurring metal-binding peptides found in plants and fungi (Rauser, 1995). However, the presence of a γ bond between amino acids makes it difficult to synthesize a large quantity of PCs for practical applications. Our approach is to construct synthetic genes coding for protein analogs of PC and to investigate their capability to bind heavy metals such as cadmium. We demonstrated that even synthetic phytochelatins with up to 20 cysteines (EC20) can bind cadmium with very high affinity. The measured Cd^{2+} binding stoichiometry of 10 was 40% higher than the value reported for MTs. This result represents the first reported metal-binding experiment with any PC or EC with more than 10 cysteines.

Enhanced Cd^{2+} accumulation by genetically engineered *E. coli* with surface-expressed metal-binding peptides have been reported by others (Sousa et al., 1996, 1998; Xu and Lee, 1999). One of the most successful is the LamB fusion system, which has been used to anchor MTs and short metal-binding peptides onto the surface of *E. coli* (Kotrba et al., 1999; Sousa et al., 1998). However, the Cd^{2+} binding capability of cells expressing EC20 is almost twice the amount obtained using the LamB system . Even cells expressing EC11 can accumulate similar values of Cd^{2+}. Two

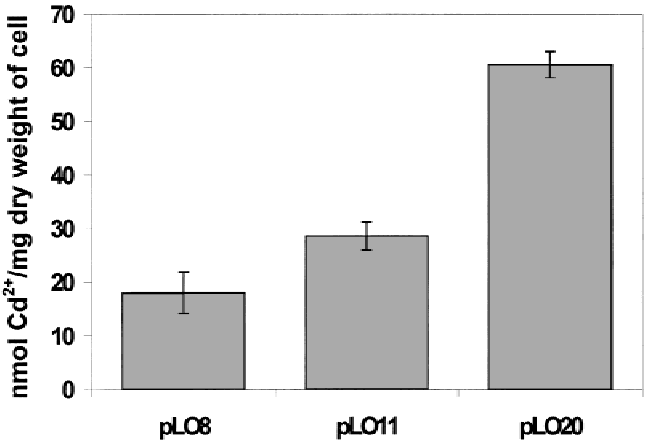

Figure 6. Effect of the chain length of EC on the bioaccumulation of Cd^{2+}. The date were obtained from five independent experiments.

factors can potentially contribute to this difference in metal accumulation. First, metal accumulation is a strong function of the binding affinity of surface-exposed peptides. Since the N- and C-terminals of the metal-binding proteins were sandwiched between the LamB fusion, they may lack the required flexibility of achieving the specific affinity for heavy metals. This is especially important if the cooperation between neighboring amino acid functional groups plays an important role in metal binding or metal binding actually induces the folding of peptides (as in the case of MTs and ECs). Since only the N-terminus end of the ECs is attached to Lpp-OmpA, this fusion system is superior to LamB in allowing the required flexibility of achieving the specific affinity for heavy metals. The increase in whole cell accumulation may also reflect the higher Cd^{2+} binding stoichiometry for EC20 than MTs. For any practical application, it is advantageous for an organism to produce 40 amino acid-long ECs containing 20 cysteines than using 60 amino acid-long MTs containing the same number of cysteines.

The second factor that influences whole-cell accumulation of Cd^{2+} accumulation is the amount of peptides expressed on the surface. In contrast to the results reported by Valls et al. (1998), who failed to detect the expression of MT as a fusion with Lpp-OmpA, we were successful in expressing ECs on the cell surface. The expression level of the different ECs was very similar, illustrating that larger peptides have very limited impact on expression. The versatility of the Lpp-OmpA in targeting proteins of different sizes is well documented (Francisco et al., 1992, 1993; Richins et al., 1997). Owing to their very high affinity to metals and the high level of expression, cells expressing Lpp-OmpA-EC fusions may be very useful as bioadsorbents for heavy metal removal. It is possible that even ECs with higher than 20 cysteine can be used for metal binding. The utility of immobilized whole cell for heavy metal removal is currently under investigation.

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