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Enhanced Bioaccumulation of Heavy Metal Ions by Bacterial Cells Due to Surface Display of Short Metal Binding Peptides

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Metal binding peptides of sequences Gly-His-His-Pro-His-Gly (named HP) and Gly-Cys-Gly-Cys-Pro-Cys-Gly-Cys-Gly (named CP) were genetically engineered into LamB protein and expressed in *Escherichia coli*. The Cd^{2+}-to-HP and Cd^{2+}-to-CP stoichiometries of peptides were 1:1 and 3:1, respectively. Hybrid LamB proteins were found to be properly folded in the outer membrane of *E. coli*. Isolated cell envelopes of *E. coli* bearing newly added metal binding peptides showed an up to 1.8-fold increase in Cd^{2+} binding capacity. The bioaccumulation of Cd^{2+}, Cu^{2+}, and Zn^{2+} by *E. coli* was evaluated. Surface display of CP multiplied the ability of *E. coli* to bind Cd^{2+} from growth medium fourfold. Display of HP peptide did not contribute to an increase in the accumulation of Cu^{2+} and Zn^{2+}. However, Cu^{2+} ceased contribution of HP for Cd^{2+} accumulation, probably due to the strong binding of Cu^{2+} to HP. Thus, considering the cooperation of cell structures with inserted peptides, the relative affinities of metal binding peptide and, for example, the cell wall to metal ion should be taken into account in the rational design of peptide sequences possessing specificity for a particular metal.

During the last few decades extensive attention has been paid to the hazards arising from contamination of the environment by heavy metals (35). Decontamination of heavy metals in the soil and water around industrial plants has been a challenge for a long time. The use of microorganisms for the recovery of metals from waste streams (15, 17, 30, 31), as well as the employment of plants for landfill application (47), has achieved growing attention. Lower cost and higher efficiency at low metal concentrations make biotechnological processes very attractive in comparison to physicochemical methods for heavy metal removal (17).

The microbial processes for bioremediation of toxic metals and radionuclides from waste streams employ living cells, nonliving biomass, or biopolymers as biosorbents (17, 30, 46). Specific metabolic pathways resulting in bioprecipitation of heavy metals or their biotransformation to less toxic or easily recoverable forms have been described (15, 17, 30, 31). A wide variety of fungi, algae, and bacteria are now under study or are already in use as biosorbents for heavy metal remediation (17, 30, 46). Metal binding by biomolecules of structural components or excreted polymers is fortuitous, and relative efficiencies depend on attributes of the metal ion, as well as on the reactivity of the provided ligands. The macromolecular composition of biosorbent could be manipulated by cultivation conditions (e.g., stress-inducible fungal melanins [30]) to improve its metal binding properties.

The principles governing the selectivity of biomolecules for metal ions are described by semiempirical and qualitative theories, such as the HSAB (hard and soft acids and bases) principle and the Irwing-Williams series of stability constants for divalent ions (26). Anchoring of particular amino acid sequences to biosorbent material could contribute to the selectivity for specific metal ions. Biosorbents could be enriched with amino acids classified by HSAB principles to be stronger ligands of transition metals than those naturally present on the microbial surfaces (26, 30). The principal benefit of selectivity should provide preference of particular metal ions for its specific coordination preferences (1), exploiting peptides with known fold. Surface exposure of metal binding peptides could improve metal binding properties of microorganisms employed in various systems based not only on biosorption but also on the metabolic activities located on the cell surface (15, 31).

A number of vehicles, including subunits of cellular appendages or outer membrane proteins, are now in use for the display and action of enzymes, peptide libraries, antigenic determinants, or single-chain antibodies on the surface of gram-negative bacteria (19). The *Escherichia coli* maltoporin (LamB) has been well characterized. The LamB protein is a trimeric outer membrane (OM) protein of *E. coli* sustaining two biological functions. It is used as a surface receptor by a number of coliphages, including phage λ (6, 12, 14), and participates in the transport of maltose and maltodextrins across the OM (45). LamB tolerates insertions of long heterologous peptides at a permissive loop (between structural codons 153 and 154) exposed to the external medium without a loss of function (5, 9, 11, 13, 21, 43, 44). Successful attempts to introduce polyhistidine tails (41), as well as yeast and human metallothioneins (42), to LamB have been reported. The surface display of the polyhistidine tail and/or metallothioneins led to a significant increase in the accumulation of divalent heavy metal ions. The LamB protein was also used to search for repeating peptides responsible for a specific adhesion of *E. coli* to gold, chromium, or iron oxide (7, 8).

We examined metal binding properties of *E. coli* strains displaying short peptides as a fusion to LamB protein. The histidine-rich sequence Gly-His-His-Pro-His-Gly employed in this study was named HP. HP represents one to three multiple repeats along the C-terminal part of the human plasma metal transport protein known as the histidine rich-glycoprotein

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(HRG) (28). The HRG binds heme and various divalent heavy metal ions with the following apparent order of affinity: $Cu^{2+} \sim Hg^{2+} > Zn^{2+} > Ni^{2+} > Cd^{2+} > Co^{2+}$ (35). The HP sequence is believed to form surface metal binding sites (MBSs) of HRG, and it has been also successfully used to immobilize Cu^{2+} and Zn^{2+} on IMAC columns (27). The cysteine-rich amino acid sequence, named CP (Gly-Cys-Gly-Cys-Pro-Cys-Gly-Cys-Gly), was previously selected in our laboratory as a result of screening of synthetic peptides consisting of cysteine and histidine residues for Cd^{2+} binding (29). CP was further characterized and employed for display on the *E. coli* surface.

MATERIALS AND METHODS

Chemical synthesis of peptides. The standard Merrifield solid-phase technique with diisopropylcarbodiimide–1-hydroxybenzotriazole activation chemistry was used for the synthesis of the CP peptide of the sequence AcOGly-Cys-Gly-Cys-Pro-Cys-Gly-Cys-resin and the HP peptide of the sequence AcOGly-His-His-Pro-His-Gly-resin. The peptides were synthesized on TentaGel resin without cleavable linker (Rapp polymere). FMOC (9-fluorenylmethoxy carbonyl)-tertiary butyl-protected amino acids (Senn Chemicals) were added to the peptide-conjugated resin in a threefold molar excess to amino groups at a concentration of 0.3 M. Coupling was continued for 1 h, and each position was doubly coupled. The FMOC group was deprotected with 20% piperidine-dimethylformamide (the first treatment was for 2 min, followed by the second for 20 min). After coupling of the last amino acid, the N terminus was acetylated. The deprotection of the peptide side chains was accomplished by treatment with 90% trifluoroacetic acid–2.5% thioanisole–2.5% ethanedithiol–2.5 triisopropylsilane–2.5% water for 2 h. The resin was then washed and neutralized with 10% diisopropylethylamine (in dimethylformamide). Peptides were subjected to amino acid analysis in order to verify the amino acid composition of peptide and to determine amount of peptide on carrier. The sulfhydryl groups of CP peptide were determined by using a reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (37). An appropriate amount of TentaGel with CP peptide was incubated with 1 mM DTNB in 0.1 M phosphate buffer (pH 7.27) for 1 h with shaking. The absorption was read at 412 nm (ε_{412} = 13,700 M^{-1} cm^{-1}) after the completed reaction.

Strains, plasmids, general procedures, and media. *E. coli* TG1 (*supE hsdΔ5 thi* Δ[*lac-proAB*] F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]) was used to host and multiply recombinant plasmids. The *lamB* mutant *E. coli* strain pop6510 (*supE thr leu tonB thi lacY1 recA dex5 metA*) was used as a recipient of all expression vectors bearing LamB variants. The R type of lipopolysaccharide (LPS) of this strain was determined by silver staining (21). *E. coli* LE392 (*supE supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1*) was used to propagate λ phages. Lambda phages λh$^+$ (wild type), λh^0 (single mutant), and λhh* (double mutant) have been described elsewhere (6, 12). The vector pLBB9 used for the expression of LamB variants has been described previously (9). pLBB9 is a derivative of pSC101-based, chloramphenicol (Cm)-resistant vector pVDL8 carrying a promoterless *lamB-153* gene that is expressed through the *Plac* promoter of the vector. Insertions between the positions 153 and 154 of the amino acid sequence of LamB protein have been constructed via a unique *Bam*HI site of the plasmid. Recombinant DNA techniques were carried out according to a standard protocol (38). The orientation of the insertions was verified by restriction analysis, and positive clones were subjected to DNA sequencing.

Minimal MJS medium (12.5 mM HEPES, pH 7.1; 50 mM NaCl; 20 mM NH_4Cl; 1 mM KCl; 1 mM $MgCl_2$; 0.1 mM $CaCl_2$; 0.05 mM $MnCl_2$; 0.8% Casamino Acids; 0.4% glycerol; 0.005% thiamine) and complete Luria-Bertani (LB) medium were supplemented with 30 μg of Cm per ml and 10 or 100 μM IPTG (isopropyl-β-D-thiogalactopyranoside) when required.

Construction and expression of hybrid LamB proteins. Two complementary pairs of oligonucleotides encoding peptide insertions to *lamB-153* were designed. Both pairs were flanked at 5′ and 3′ ends by *Bam*HI and *Bgl*II cohesive termini, respectively. Such a design allows specific insertions into a unique *Bam*HI site of the *lamB-153* gene which reconstitute only one *Bam*HI site (at the 5′ end) and allows successive insertions of another DNA fragment in tandem. The 5′-GATCCAGCTGGTCATCATCCACACGGTGCT-3′ (plus strand) encodes the N-Ala-Gly-His-His-Pro-His-Gly-Ala-C sequence, which has been named HP. The 5′-GATCCAGCAGGCTGCGGTTGTCCATGCGGTTGTGGCGCT-3′ (plus strand) encodes the N-Ala-Gly-Cys-Gly-Cys-Pro-Cys-Gly-Cys-Gly-Ala-C sequence, which has been named CP. By using this approach, DNA fragments encoding HP peptide and CP peptide were inserted into *lamB-153* of the vector pLBB9, resulting in *lamB-HP* (plasmid pLBHP) and *lamB-CP* (plasmid pLBCP), respectively. Subsequently, the next DNA fragments encoding HP peptide and CP peptide were inserted into *lamB-HP*, resulting in *lamB-HP2* (plasmid pLBHP2) and *lamB-HPCP* (plasmid pLBHPCP), respectively (see Fig. 2a).

Expression of LamB derivatives (those with a genetic insertion of a metal binding site are further called LamB-MBS) was performed in MJS or LB media supplemented with 30 μg of Cm per ml and 100 μM IPTG.

Sensitivity of *E. coli* to λ phage variants. High-titer phage lysates were prepared by infection and lysis of the permissive strain *E. coli* LE392 as reported elsewhere (40, 42). Approximately 100 μl of lysate (titer of ~10^{10} PFU/ml) was streaked in a line across the surface of LB agar plates (supplemented with 5 mM [each] $CaCl_2$ and $MgSO_4$, 30 μg of Cm per ml, and 10 μM IPTG) and allowed to dry. *E. coli* pop6510 bearing each of the plasmids encoding LamB variants was then streaked perpendicular to and across the phage line in a single swatch. The sensitivity was evaluated after overnight incubation at 37°C.

Preparation of *E. coli* envelopes. The method for the preparation of cell envelopes of *E. coli* for metal binding studies has been described elsewhere (2). Briefly, *E. coli* pop6510 expressing LamB or LamB-MBSs was harvested from 500 ml of LB media supplemented with 30 μg of Cm per ml and 100 μM IPTG. The pellet was resuspended in 3 ml of 50 mM HEPES (pH 7.2), and the cells were disintegrated by using X-press (LKB, Stockholm, Sweden). Unbroken cells were removed by low-speed centrifugation (1,500 × *g* for 10 min at 4°C). Supernatant (disrupted cells) was incubated with RNase (100 μg/ml) and DNase (50 μg/ml) in the presence of $MgCl_2$ (5 mM). Envelopes were separated at 48,000 × *g* for 30 min at 4°C, washed five times with 6 ml of ice-cold demineralized water, and then freeze-dried.

We also used Percoll gradient separation as described before (33) for the small-scale preparation of OM and inner membrane (IM) fractions in order to determine the localization of LamB-MBS proteins.

Protein techniques. Whole-cell extracts and/or equivalent portions of cell envelopes, membrane preparations, or cytoplasmic fractions were examined by electrophoresis in a denaturing polyacrylamide gel. Proteins were alternatively electroblotted on nitrocellulose membranes blocked with 10% skim milk in TBS (20 mM Tris-Cl, pH 7.4; 250 mM NaCl; 3 mM KCl) for 1 h. Anti-LamB serum (a kind gift of M. Hofnung) preadsorbed with cell extract of *E. coli* pop6510 was applied at a 1:2,000 dilution in TBST (TBS with 0.1% Tween 20) with 2% skim milk for 2 h. Membranes were washed with TBST and incubated with goat anti-rabbit antibody conjugated with alkaline phosphatase added at a 1:5,000 dilution in TBST with 2% skim milk. Membranes were washed with TBST, and LamB variants were visualized by using 5-bromo-4-chloro-3-indolyl phosphate as a substrate along with nitroblue tetrazolium.

Metal binding studies. Cd^{2+}-to-HP and Cd^{2+}-to-CP stoichiometry was determined by using synthetic peptides immobilized on TentaGel resin. Approximately 1.2 (for HP) or 0.6 (for CP) μmol of peptides was incubated at room temperature for 4 h with shaking in 1 ml of 0.1 to 5 mM $CdCl_2$ in 50 mM Tris-Cl (pH 7.4). The constant level of ionic strength was maintained by the addition of the background electrolyte (0.2 M KNO_3) into the reaction mixture in order to support saturation of HP peptide at the lower Cd^{2+} concentrations. Beads were then sedimented by gravity, and the metal concentration was determined by atomic absorption spectrometry (Varian Spectra A300).

Next, 3 mg (dry weight) of envelopes was incubated at room temperature for 30 min in 3 ml of 5 mM $CdCl_2$ in 25 mM HEPES (pH 7.0) in order to determine the extent of Cd^{2+} binding by *E. coli* envelopes containing LamB-MBSs. Envelopes were pelleted at 40,000 × *g* for 30 min at 4°C and then washed five times with 3 ml of ice-cold 25 mM HEPES (pH 7.0). Envelopes were then mineralized with 70% nitric acid overnight under atmospheric pressure at room temperature. The mineralized product was then diluted with dimineralized water, and precipitated proteins were removed by centrifugation. The metal concentration was determined by atomic absorption spectrometry.

Bioaccumulation of metals (Cd^{2+}, Cu^{2+}, and Zn^{2+}) was measured in cells growing in MJS medium with Cm. The low-phosphate MJS medium (employed in order to avoid the precipitation of heavy metals) was supplemented with the heavy metal of interest at a nontoxic concentration. The metal chlorides were used in order to prefer biosorption of metal on the cell surface to its intracellular uptake (18). We did not detect any precipitation of heavy metals in the media or any measurable sorption of metals on the glassware surface under these conditions. The cells were induced with IPTG (100 μM) at an optical density at 590 nm (OD_{590}) corresponding to 0.3. The metal chloride(s) added up to a total concentration of 30 μM (i.e., either a 30 μM concentration of a single metal or a 15 μM concentration of each metal in a double-metal assay) at an OD_{590} of 0.4. The cultures were grown for another 3.5 h. Prior to the determination of the metal content, the cells were pelleted, washed twice with 0.85% NaCl in 5 mM HEPES (pH 7.1), and then mineralized overnight with 70% nitric acid. Mineralized cells were further treated as described above. Alternatively, washed cells were incubated for 15 min with an excess volume of ice-cold 5 mM EDTA in 0.85% NaCl (pH 7.1) in order to remove the surface-bound metal. The cells were then pelleted and treated as described above.

RESULTS

Stoichiometry of Cd^{2+} binding to synthetic peptides. Two peptides predicted to be candidates for the engineering of bacterial surface for enhanced heavy metal binding were synthesized. The amino acid sequences were Gly-His-His-Pro-His-Gly (i.e., HP) and Gly-Cys-Gly-Cys-Pro-Cys-Gly-Cys-Gly (i.e., CP). The correct amino acid composition and the amount of peptide bound on TentaGel resin were evaluated by amino

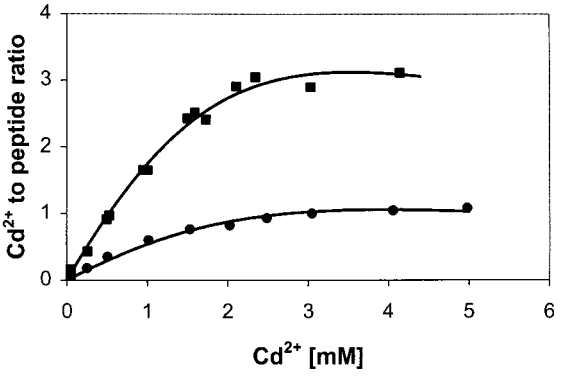

FIG. 1. The Cd^{2+}-to-peptide stoichiometry expressed as the plot of initial Cd^{2+} concentration against the complexed Cd^{2+}-to-peptide molar ratio. A total of 0.6 μmol of CP peptide (squares) and/or 1.2 μmol of HP peptide (circles) were incubated on TentaGel resin in 1 ml of Cd^{2+} containing 50 mM Tris-Cl (pH 7.4). In the case of HP peptide metal binding studies, 0.2 M KNO_3 was added as a background electrolyte. The portion of unbound Cd^{2+} was determined by atomic absorption spectrometry.

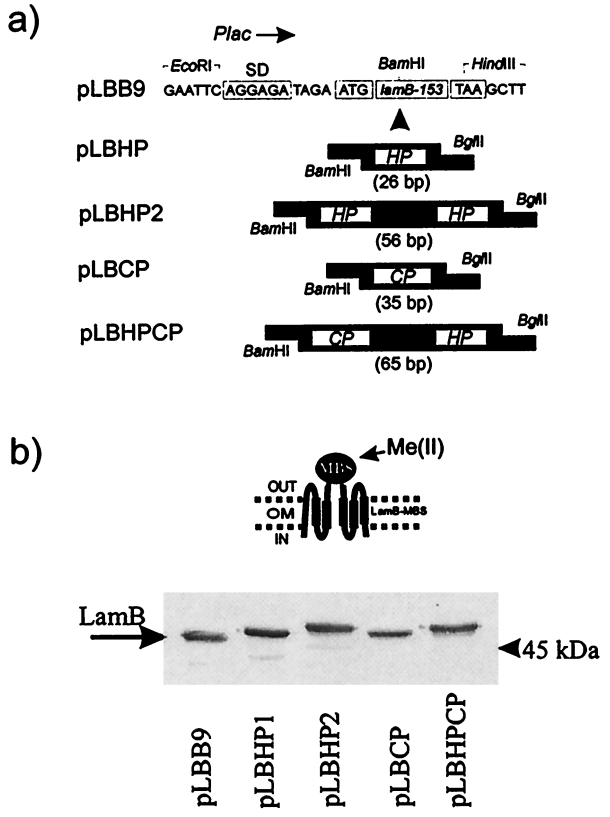

FIG. 2. (a) Organization of the *lamB-mbs* gene within the pLBB9 expression vector (a derivative of the low-copy-number vector pVDL8 bearing *lamB-153* gene expressed throughout the *lac* promoter). The orientation of the promoter is marked by an arrow. The ribosome binding site (SD), the initiation codon (ATG), and the stop codon (TAA) of *lamB-153* are indicated. The plasmids relevant to the specific genetic insertions of *mbs* indicated are listed on the left. For the amino acid compositions of the MBSs see Table 1. (b) Expression of LamB-MBD in *E. coli* pop6510. Crude extracts of approximately 2×10^8 cells of *E. coli* pop6510 expressing LamB variants were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, probed with preadsorbed polyclonal rabbit anti-LamB serum, and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase. The arrow indicates the position of wild-type LamB protein. The drawing shows the desired targeting of LamB-MBSs into the OM of *E. coli*. The LamB protein consists of 18 transmembrane domains, and the MBSs are introduced between transmembrane domains 7 and 8 of the protein.

acid analysis after total peptide hydrolysis. The sulfhydryl content of CP peptide was also determined. These analyses confirmed that peptides were synthesized as correct full-length sequences. The amounts of peptide were found to be 138 and 118 nmol per mg of carrier for HP and CP peptide, respectively. The yield represented approximately 50% of the theoretical yield.

The Cd^{2+}-to-peptide stoichiometry was determined from the plot of the initial metal concentration against the molar ratio of bound cadmium to peptide (Fig. 1). The resulting Cd^{2+}-to-HP peptide stoichiometry of 1:1 indicated the presence of a single metal binding site. On the other hand, CP peptide was found to bind three equivalents of Cd^{2+}. These data suggested that both HP and CP peptides provide potent MBSs.

Expression of LamB hybrid proteins. DNA fragments encoding predicted MBSs were engineered into the *lamB-153* gene at a permissive position equivalent to the protein loop exposed on the cell surface (Fig. 2a). Corresponding expression vectors carrying the chimeric gene *lamB-mbs* were named pLBHP (one HP sequence), pLBHP2 (two HP sequences), pLBCP (one CP sequence), and pLBHPCP (a combination of HP and CP sequences).

The LamB-MBS proteins were expressed in *lamB*-defective *E. coli* pop6510 as full-length products, with no signs of proteolytic degradation, as demonstrated by immunoblot analysis of the crude cell extracts with anti-LamB serum (Fig. 2b). The growth rate of cells expressing LamB-MBS was not altered compared to cells expressing LamB during the exponential phase. However, a slight depression of the biomass yield (about 20%) was observed with cells expressing LamB-CP and LamB-HPCP.

Localization of LamB-MBS and functional tolerance of LamB to specific insertions of HP and CP sequences. To address the issue of the effect of insertion of HP and CP sequences and/or their combination on the targeting of LamB-MBS into outer membrane, we fractionated envelopes of *E. coli* pop6510 expressing LamB-MBS. We noted a sharp, white, high-density band resulting from Percoll gradient centrifugation of the disintegrated cells corresponding to the OM of *E. coli* (33) (Fig. 3a). A band close to the top of the gradient as a fraction consisting of the IM was also separated out. The majority of the LamB-MBS proteins were present in the fraction corresponding to the OM of *E. coli*, i.e., at the site of its natural destination (Fig. 3 shows LamB-HP2 as an example).

The sensitivity of *E. coli* pop6510 expressing LamB-MBS to lambda phages λh^+ (wild type), λh^0, and λhh* was determined in order to evaluate the effect of specific insertions (MBSs) on the folding of the LamB protein. No changes of the sensitivity to lambda phages were detected in the chimeric LamB.

Metal binding properties of isolated envelopes containing LamB-MBSs. Cell envelopes were prepared by a method described elsewhere (2). The presence of the LamB-MBSs was determined by immunoblot analysis. No significant differences in LamB-MBS content were observed among all of the preparations (data not shown). We did not detect any contamination of the envelopes with nucleic acids. The reaction of the envelopes with Cd^{2+} was performed as described in Materials and Methods in an arrangement similar to that described elsewhere (2, 23, 24), but the pH was set to 7.0. This value remained unchanged during the reaction. As shown in Table 1, *E. coli* envelopes containing any of the LamB-MBSs bound significantly higher amounts of Cd^{2+} than did those of cells

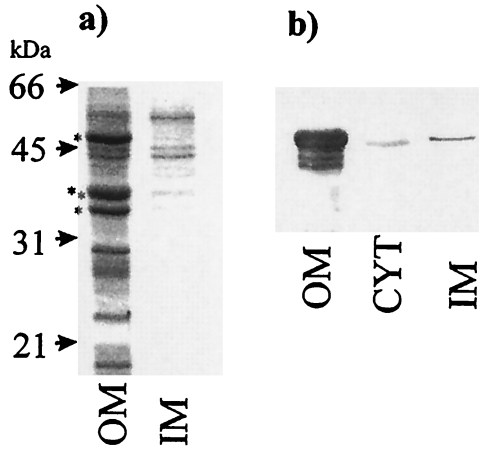

FIG. 3. Localization of LamB-HP2 protein in *E. coli*. (a) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the OM and the IM fractions resulting from Percoll gradient centrifugation. Proteins of resolved membrane vesicles of approximately 5×10^9 cells were precipitated with acetone and run in a 12% denaturing polyacrylamide gel. Asterisks indicate the major OM proteins identified on a molecular-size basis (LamB-HP2, 48.8 kDa; OmpC, 38.2 kDa; OmpF, 37.0 kDa; OmpA, 35.1 kDa). (b) Immunochemical detection of LamB-HP2 in the same membrane fractions. CYT, aliquot portion of cytoplasm resulting from high-speed centrifugation of disrupted cells.

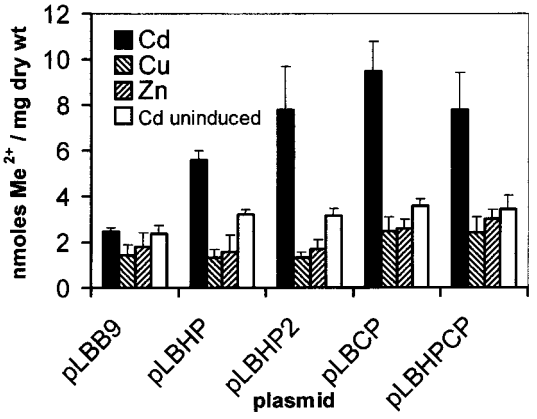

FIG. 4. Bioaccumulation of heavy metal ions by *E. coli* pop6510 expressing LamB-MBSs in a single-metal system. *E. coli* transformed with plasmids carying specific insertions in *lamB-153* (see Table 1) or the control plasmid pLBB9 was grown in MJS medium and induced at $OD_{590} = 0.3$ with IPTG except for the control uninduced cells (open bars). Cells were further grown until $OD_{590} = 0.4$, and then a 30 μM concentration of $CdCl_2$, $CuCl_2$, or $ZnCl_2$ was added. The metal content was determined by atomic absorption spectrometry after an additional 3.5 h of cultivation. The bars represent the mean value of three to five independent experiments.

expressing "wild-type" LamB protein. The best metal binding capacity showed envelopes containing LamB-HP2 and/or LamB-CP, which exceeded the natural ability of *E. coli* cell envelopes to bind Cd^{2+} by 1.8-fold.

Metal binding properties of *E. coli* displaying LamB-MBS. The increased Cd^{2+} binding capacity of the manipulated cell wall of *E. coli* led us to evaluate the influence of specific genetic insertions on metal binding by viable *E. coli* cells. The accumulation of bivalent metal ions in the "single metal" (Cd^{2+}, Cu^{2+}, or Zn^{2+}) system was compared to that of the cells expressing wild-type LamB protein.

As shown in Fig. 4, cells displaying LamB-MBSs accumulated Cd^{2+} with an efficiency higher than that of the other tested metals. Insertion of MBSs containing both histidines (HP) and cysteines (CP) into the LamB protein led to the increase of the amount of accumulated Cd^{2+} from media supplemented with 30 μM Cd^{2+}. A more than twofold increase (2.2 ± 0.3) of Cd^{2+} bioaccumulation was observed with LamB-HP. Duplication of the HP sequence (LamB-HP2) led to the additional increase (3.1 ± 1.0 times) of the amount of accumulated Cd^{2+}. A nearly fourfold increase (3.8 ± 0.8) of the Cd^{2+} bioaccumulation occurred in the cells expressing LamB-CP. However, there was no additive effect of the combination of HP and CP sequences (LamB-HPCP) on the total amount of accumulated Cd^{2+} (Fig. 4). A similar pattern was found for the accumulation of Cd^{2+} from medium supplemented with 15 μM Cd^{2+} (Fig. 5). A slight increase in the Cd^{2+} bioaccumulation by uninduced cells was observed (Fig. 4). This was due to leaking expression of LamB-MBS as detected by immunoblot.

E. coli cells preadsorbed with Cd^{2+} were incubated in excess of EDTA (see Materials and Methods) in order to evaluate the portion of Cd^{2+} bound to the surface structures. Such treatment, which may also cause a partial release of LPSs (31), resulted in removal of 50 to 60% of the total Cd^{2+} accumulated by cells expressing LamB-MBSs (Table 1). The surface display of the histidine-based MBS (LamB-HP and LamB-HP2) did not enhance the bioaccumulation of both Cu^{2+} and Zn^{2+} in contrast to the accumulation of Cd^{2+}. A slightly increased bioaccumulation of Cu^{2+} and Zn^{2+} was observed with cells expressing LamB-CP and LamB-HPCP. However, this increase was less than twice that of the control.

TABLE 1. The amount of Cd^{2+} bound by envelopes of *E. coli* pop6510 expressing LamB-MBSs and a portion of Cd^{2+} removed from the surface by EDTA treatment[a]

Plasmid transformed to *E. coli* pop6510	Amino acid sequences (MBSs) inserted into LamB protein[b]	Cd^{2+} removed by EDTA treatment (%)[c]	Cd^{2+} bound by cell envelopes (nmol/mg [dry wt])[d]
pLBB9	None	69	125
pLBHP	N-Asp-Pro-Ala-Gly-His-His-Pro-His-Gly-Ala-C	60	196
pLBHP2	N-(Asp-Pro-Ala-Gly-His-His-Pro-His-Gly-Ala)$_2$-C	59	226
pLBCP	N-Asp-Pro-Ala-Gly-Cys-Gly-Cys-Pro-Cys-Gly-Cys-Gly-Ala-C	55	227
pLBHPCP	N-Asp-Pro-Ala-Gly-Cys-Gly-Cys-Pro-Cys-Gly-Cys-Gly-Ala-Asp-Pro-Ala-Gly-His-His-Pro-His-Gly-Ala-C	51	184

[a] Amino acid sequences corresponding to DNA fragments artificially introduced into *lamB-153* (MBSs) and the names of the corresponding plasmids are indicated.
[b] The amino acids of the MBSs are underlined.
[c] Cells preadsorbed with cadmium as described in the legend to Fig. 3 were treated for 15 min with an excess volume of ice-cold 5 mM EDTA in 0.85% NaCl (pH 7.1), and the metal content was determined by atomic absorption spectrometry. The data represent the mean value of two independent experiments.
[d] The mean value of two independent experiments with a span of less than 10%. First, 3 mg (dry wt) of the cell envelopes was incubated for 30 min in 3 ml of 5 mM $CdCl_2$ in 25 mM HEPES (pH 7.0). The metal content of the envelopes was then determined by atomic absorption spectrometry.

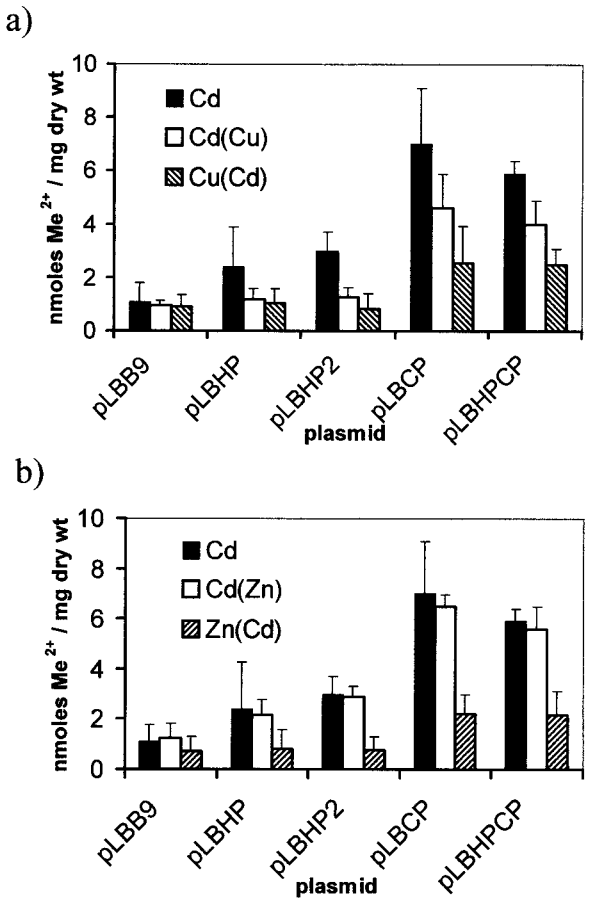

FIG. 5. Bioaccumulation of heavy metal ions by *E. coli* pop6510 expressing LamB-MBSs in the double metal system. *E. coli* transformed with plasmids carrying specific insertions in *lamB-153* (see Table 1) or the control plasmid pLBB9 was grown in MJS medium and induced at OD_{590} = 0.3 with IPTG. Cells were than grown until OD_{590} = 0.4 and supplemented with 15 μM $CdCl_2$ and/or equimolar mixtures (15 μM concentrations of each metal ion) of either $CdCl_2$ and $CuCl_2$ (a) or $CdCl_2$ and $ZnCl_2$ (b). Their metal content was determined by atomic absorption spectrometry after an additional 3.5 h of cultivation. The second metal of the mixture is indicated in brackets. The bars represent mean value of three independent experiments.

The absence of a contribution from the HP sequence for the Cu^{2+} binding was quite unexpected since the imidazolium group has been described as a ligand with a relatively high affinity for Cu^{2+} in biological systems (26). The competition of Cd^{2+} with Cu^{2+} or Zn^{2+} for LamB-MBSs in vivo was evaluated in order to elucidate this phenomenon. As shown in Fig. 5a, the amount of Cd^{2+} accumulated by cells expressing LamB-HP and LamB-HP2 from media containing an equimolar mixture of Cd^{2+} and Cu^{2+} (15 μM each) dropped to the level accumulated by control cells expressing wild-type LamB. The presence of Zn^{2+} (15 μM) caused less than a 10% decrease of bioaccumulation of Cd^{2+} by cells displaying HP sequences compared to the same cells grown in the presence of a single 15 μM Cd^{2+} (Fig. 5b). The cells expressing LamB-CP and LamB-HPCP accumulated a greater amount of heavy metals than the control from equimolar mixtures of both Cd^{2+} and Cu^{2+} or both Cd^{2+} and Zn^{2+} (Fig. 5). However, the decrease in the amount of Cd^{2+} accumulated due to the presence of Cu^{2+} in the medium was significant (Fig. 5a). An apparently lower effect of Zn^{2+} on Cd^{2+} accumulation was observed with cells displaying the CP sequence (Fig. 5b).

DISCUSSION

The introduction of additional peptides serving as heavy metal ligands on the microbial surface represents one possible way for improving the metal binding properties of the biomass in terms of capacity, kinetics, and selectivity. The metal binding ability of the *E. coli* cell wall has been studied in detail previously (2, 16, 23, 24). The specific native groups of the OM participating in metal binding are the polar head groups of phospholipids acting mainly at the inner layer of the OM and the acidic groups of the exposed (hydrophilic) polypeptides and at the outer half of the OM LPSs. The LPSs provide both carboxyl and phosphoryl groups as ligands. However, only the latter group is responsible for the high affinity of divalent metal ions for LPS (16). The peptidoglycan (PG) layer of *E. coli*, which is most probably one molecule thick, binds metal ions via the carboxyl group of the D-glutamic acid of the peptide stem and the hydroxyl groups of the glycan backbone (23). The two-step deposition process may increase the apparent metal binding capacity of PG (3, 23) and would include the stoichiometric binding of metal ion, generating a nucleation site for the subsequent precipitation of metal above the stoichiometric amounts.

The OM LamB protein of *E. coli* has been reported to tolerate the genetic insertions of heterologous peptides at positions between the structural codons 153 and 154 (5, 9, 11, 13, 22, 42, 43, 44). The genetic insertions of the HP and CP sequences into *lamB-153* resulted in LamB-MBS hybrid proteins located in the OM. Furthermore, the LamB-MBSs retained their biological function as a lambda phage port, indicating a maintenance of their overall folding pattern. The Cd^{2+}-to-peptide stoichiometry determined for synthetic HP and CP peptides is less than one metal ion per amino acid residue possessing metal binding properties (Fig. 1). This suggests specific folding of the sequence around the metal ion(s). The geometry of the MBSs of both HP and CP peptides is currently under study.

The increase of the metal binding capacity of the *E. coli* envelopes was significant (Table 1). Thus, the introduction of metal binding peptides onto the surface of the microorganism to be used as nonliving material for bioremediation may improve the process. It could be hypothesized that such an approach will improve not only the metal binding capacity of the biosorbent but also the kinetics of the process. An appropriate carrier for the surface display could be the only limiting factor. The C-terminal part of α-agglutinin could be such a carrier in yeast cells (39), which are being considered for use in the bioremediation of heavy metal ions (25).

We used *E. coli* as a model to evaluate changes of the metal binding properties due to surface display of the metal binding peptides. Surface display of HP and CP sequences resulted in a significant increase in Cd^{2+} bioaccumulation by growing *E. coli* (Fig. 4 and 5). The number of Cd^{2+} binding sites generated by surface display of polyhistidine or metallothionein did not fully account for the amount of accumulated Cd^{2+}, as has been previously proposed (41, 42). The amount of LamB used in the expression system ranged from 1,000 to 5,000 molecules of protein per cell. This value is 2 to 3 orders of magnitude lower than the increment in Cd^{2+} accumulation due to the insertion of the MBS. It has been suggested that displayed peptides favor the interaction of Cd^{2+} with other bacterial structures by increasing the local concentration of metal ions (41, 42). The cell wall components involved could be, for example, LPSs (*E. coli* pop6510 possesses the R type of LPS), which are known as compounds that directly interact with the LamB protein (36) and provide sufficiently effective metal ligands (16). The data

on desorption-bound Cd^{2+} by EDTA treatment suggest that more than one-half of the metal is located on the surface.

A different situation has been observed for Cu^{2+} and Zn^{2+} bioaccumulation. While *E. coli* displaying CP peptide (as LamB-CP or LamB-HPCP) accumulated both Cu^{2+} and Zn^{2+} in apparently higher amounts than did the control cells expressing LamB protein, the surface display of HP did not promote any increase of bioaccumulation of these two metals (Fig. 4). The apparent lack of contribution of the HP sequence was unexpected because histidine residues possess a higher affinity to Cu^{2+} than to Cd^{2+} (26, 34) and because the HP sequence by itself has been shown to be an effective ligand for both Cu^{2+} and Zn^{2+} (27). We also did not detect any contribution of HP display to the enhanced bioaccumulation of Ni^{2+} and Co^{2+} (unpublished observations). Moreover, the presence of Cu^{2+} in the medium resulted in the inhibition of Cd^{2+} binding by cells displaying HP peptide. This finding confirms the strong binding of Cu^{2+} to the HP sequence in vivo. However, no increase of the Cu^{2+} accumulation by corresponding cells was observed. In adopting a model explaining the disproportions between the number of MBSs and the amount of accumulated Cd^{2+} (41, 42), it should be considered that such a figure could be due to the lower affinity of bacterial surface components other than that of HP to Cu^{2+}. The lack of increase in bioaccumulation of Zn^{2+} by cells displaying HP sequences can be explained by the low relative affinity of cell wall components for Zn^{2+}, which is otherwise bound to HP sequence less avidly than Cd^{2+} (Fig. 5b). Since the affinity of Zn^{2+} to HRG is higher than that of Cd^{2+} (33), the absence of any significant effect of Zn^{2+} on the bioaccumulation of Cd^{2+} due to HP display is quite interesting. It could indicate a different conformation of the HP sequence fused to LamB from that in HRG or the participation of other amino acid residues in the formation of the metal (Zn^{2+}) binding site of HRG.

E. coli displaying the CP peptide (either as LamB-CP or LamB-HPCP) exhibited an accumulation of both Cd^{2+} and its counterpart in the equimolar mixture (Cu^{2+} or Zn^{2+}) higher than that of the control cells. The results shown in Fig. 5 are in agreement with the relative affinities of tested metals to imidazolium and sulfhydryl groups (26).

The LamB protein is a very attractive "broad-range" vehicle that could be efficiently expressed in various gram-negative species (9, 14, 43, 44). The engineering of metal binding peptides on the surface of environmentally acceptable gram-negative bacteria such as *Ralstonia eutropha* and *Pseudomonas putida*, which are already employed in existing systems for heavy metal bioremediation (15, 30), represents a possible application. For instance, the metal binding peptides introduced on the surface of *R. eutropha* may aid in the process of precipitation and crystallization of metal carbonates.

The search for novel peptide sequences with attention paid to their selectivity for specific metal ions is under study in our laboratory. Several designs for artificial heavy metal binding sites have been previously reported in the literature. The synthetic peptide Boc-Cys-Pro-Leu-Cys-OMe, designed as a model for Cys-containing metal binding sites, has been shown to bind Hg^{2+}, Zn^{2+}, and Cd^{2+} via both Cys residues (48). The Zn^{2+} binding via His and Glu residues was described for a model peptide mimicking the metal binding site of the ribonucleotide reductase (49). Haymore et al. (20) identified several short chelating sequences containing His, Cys, and Asp residues which could form energetically stable chelating sites with specific metal ions. Amino acid sequences forming a stable coordination sphere around transition metals were also identified by using a combinatorial peptide library approach (4). However, the data shown in Fig. 4 and 5 indicate that not only would the affinity of a peptide or its selectivity determine the bioaccumulation of a particular metal ion but that the "reactivity" of the cell wall (and cell compartments) would also have to be taken into account.

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