# Enhanced bioadsorption of cadmium and nickel by *E. coli* displaying a metal binding motif using CS3 fimbriae

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***Abstract***
*Display of peptides on the surface of bacteria offers many new and exciting applications in biotechnology. Fimbriae is a good candidate for epitope display on the surface of bacteria. The potential of CS3 fimbriae of enterotoxigenic E. coli as a display system has been investigated. A novel cell surface display system with metal binding property was developed by using CS3 fimbriae. Short metal binding peptide, Gly-Cys- Gly-Cys-Pro- Cys- Gly- Cys- Gly as a cysteine rich peptide, was inserted into CS3 fimbriae and displayed on the surface of E. coli. Bacteria expressing hybrid pili with cysteine rich peptide could adsorb 392.5, 510 and 905 nmol of $Ni^{2+}$, $Cd^{2+}$ and $Pb^{2+}$ per mg (dry weight) of cells, respectively, which are five-fold (nickel) and three-fold (cadmium) more than E. coli expressing native pili. Thus, expression of Cys-rich peptide enables bacteria to act as a metalloaffinity adsorbent. These results open the possibility for biosorption of heavy metal ions using engineered microorganisms.*
***Keywords:*** *Bacterial surface display; CS3 pili; Biosorbent; Nickle; Cadmium; Heavy metals; Cysteine-rich metal binding peptide*

## INTRODUCTION

Increase in industrial activities has intensified environmental pollution and deterioration of some ecosystems, with accumulation of pollutants such as heavy metals (Veglio and Beolchini, 1997). The use of microorganisms for sequestration of various heavy metals has been extensively studied (Pazirandeh *et al*., 1998; Gadd, 1992). Lower cost and higher efficiency of the biological heavy metal uptake from the effluents with low metal concentrations make biotechnological processes very attractive in comparison to physico-chemical methods for heavy metal removal (Kotrba *et al*., 1999). Proteins such as metallothionein or low molecular weight Cys- rich peptides can bind metal ions (e.g., $Zn^{2+}$, $Cd^{2+}$, $Cu^{2+}$, $Hg^{2+}$ and $Ag^{2+}$) and sequester them in a biologically inactive form (Sousa *et al* ., 1998).

Bacteria contain a wide range of proteins on their cell surface. These proteins are involved in a number of natural processes such as adhesion, colonization, motility, signal transduction. Fimbriae are particularly attractive candidates for epitope display. They are composed of hundreds of identical subunits present in extremely high numbers on the cell surface and can be easily purified (Klemm and Schembri, 2000). A suitable system for display of heterologous peptides is based on CS3 fimbriae. CS3 tolerates insertion of heterologous peptides at permissive sites exposed to the external medium without a loss of function (Yakhchali and Manning, 1997). Two expose regions of major subunit of CS3 (CstH) have been used for insertion and expression of the epitopes of nonstructural glycoprotein NSP4 of Rotavirus, B subunit of LT (heat labile toxin) and the entire coding sequence of mature ST toxin on the surface of *E. coli* as hybrid pili (Yakhchali and Manning, 1997; Hosseini, 2003).

In this study, a cysteine-rich metal binding peptide (Gly-Cys-Gly-Cys-Pro-Cys-Gly-Cys-Gly) (Kotrba *et al*., 1999) was displayed on the *E. coli* surface using CS3 fimbriae and the metal binding properties of recombinant bacteria was examined.

## MATERIALS AND METHODS

**Enzymes and chemicals:** All restriction enzymes and T4 DNA ligase were obtained from Roche (Germany)

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and Cinagene (Iran). *Pfu* DNA polymerase was from Fermentas (Cinagene, Iran). Primers were synthesized by MWG Biotech Company (Germany). High pure PCR product purification and high pure plasmid extraction kits were from Roche. All of the chemical reagents were obtained from Sigma (USA).

**Bacterial strain and plasmids:** Plasmids pPM4556, containing mutant *cstH* (major fimbrial subunit of CS3 pili), pPM484 and pPM4567 consist of *cst* operon were used for construction and expression of hybrid pili (Yakhchali and Manning, 1997). *E. coli* K12 TG1 strain was used as the recipient strain of all plasmids (NIGEB, Iran).

**Microbial cultures and general procedures:** The LB (Luria-Bertani) and CFA medium consist of (g/l) casamino acid, l0; Bacto yeast extract, 1.5; $MgSO_4$. $3H_2O$, 0.05; $MnCl_2$. $4H_2O$, 0.005; supplemented with 100 mg/ml ampicilin, were used for growth of bacteria and expression of hybrid CS3 pili respectively. DNA manipulations were carried out according to standard protocols (Sambrook and Russel, 2001).

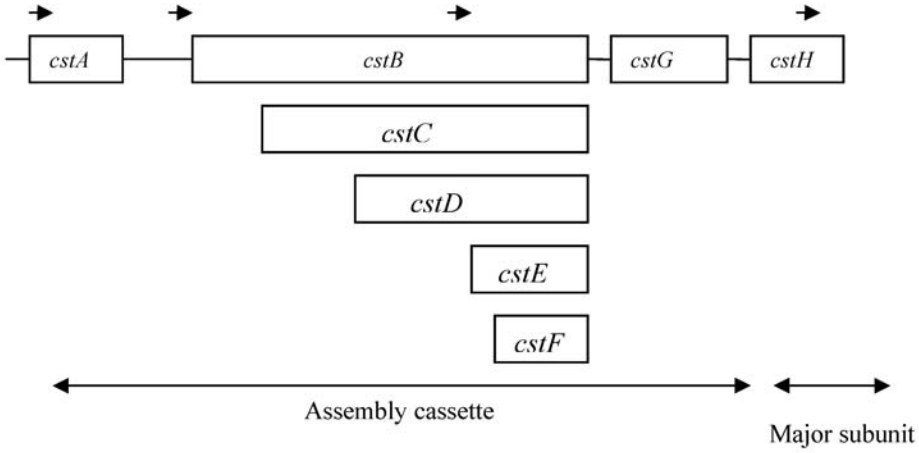


**Figure 1.** Genetic organization of the *cst* locus encoding biosynthesis and assembly of CS3. The arrows show direction of promoters (Yakhchali and Manning, 1997).

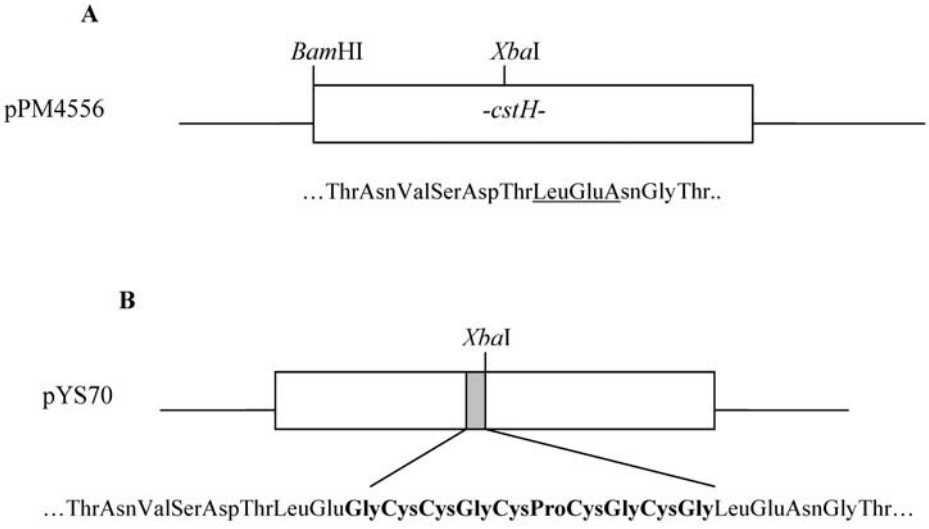


**Figure 2.** Construction of the epitope cassette encoding cysteine rich peptide. (A) pPM4556 encoding mutant CstH with 144 amino acids. A unique *Xba*I site has been introduce into *cstH* using site directed mutagenesis (Yakhchali and Manning, 1997). (B) Recombinant plasmid pYS70 consisted of hybrid gene. MPB sequence was introduced into the *XbaI* site of the *cstH* in the pPM4556. Amino acid sequence of MBP in the engineered protein has been shown.

**Construction of hybrid gene:** Genetic organization of the *cst* operon encoding biosynthesis and assembly of CS3 is shown in Figure 1. A 650 bp DNA fragment encoding mutant *CstH* has been cloned in pPM4556 (Yakhchali, 1996) (Fig. 2). Oligonucleotide B-Metal (CTAG*TCTAGA*CCACAACCGCATGGACAAC-CGCAGCC*GAGCTC*TG TATCAGAAACATTTGTACTC) containing *Xba*I at the 5´ end and *Sac*I sites corresponding to nucleotide 260-282 of *cstH* was designed to allow PCR amplification of a DNA fragment encoding the N-terminal of the CstH (65 aa) with the Cys-rich peptide between amino acids 65 and 66 of CstH. The PCR reaction was performed in 25 µl total volume using *pfu* DNA polymerase (1 unit) and its buffer, 0.2 mM dNTP, 10 µg template DNA and 10 pmol of each of the M13 forward and B-Metal primers. The PCR was performed by 5 min at 95ºC and then 30 cycles of 1 min at 95ºC, 30 s at 52ºC and 45 s at 72ºC. The PCR product was purified, digested with *Bam*HI and *Xba*I, and ligated into pPM4556 previously digested with the same enzymes and transformed into *E. coli* TG1 strain. Dideoxy Termination sequencing with the ABI automated sequencer was used to confirm the authenticity of the constructed gene.

Plasmid pPM4567 containing *cst* operon and recombinant plasmid carrying CstH::Cys-rich peptide coding DNA were cleaved with *Aoc*I and *Bam*HI enzymes. The DNA fragment containing assembly genes of *cst* operon was isolated and recovered from the low melting agarose gel and then ligated to the hybrid gene to produce hybrid *cst* operon. Clones were screened by digestion with *Aoc*I/*Bam*HI enzymes.

**Pili preparation:** The pili was prepared by the method described previously (Yakhckali, 1996) with minor modifications. The bacteria from an overnight culture on CFA agar (Colonization Factor Antigens), containing the appropriate antibiotic, were harvested into 1 ml PBS and incubated at 56ºC, for 20 min. The cells were harvested by centrifugation at 13000×g for 10 min. Trichloro acetic acid was added to the supernatant at a final concentration of 12% v/v and incubated on ice for 1h. The pili were precipitated by centrifugation at 18000×g for 15 min and stored at -20ºC.

**Production of polyclonal antibody against CS3 pili:** The pili prepared from the bacteria were separated by preparative SDS-PAGE. The area containing pili was excised from the gel and then eluted into 0.05 M Tris-HCl buffer. Protein concentration was determined according to the Bradford method. A polyclonal antiserum against the purified pili was prepared in N.Z. white rabbit. The first inoculum of the protein was prepared with emulsification of 100 µg of the protein in Freund's complete adjuvant (Sigma, USA). All the subsequent inoculation was prepared in Freund's incomplete adjuvant. Rabbit was injected subcutaneously into 2-4 sites on the animal's back (0.1-0.2 ml/site). Boosting doses of emulsified antigen were given every 4 weeks. The specificity of the polyclonal antibody was confirmed by the western blotting.

**Expression of recombinant hybrid pili:** The recombinant hybrid pili were detected by western blotting using rabbit polyclonal antiserum against CS3 pili. The purified pili was separated by 13% SDS-PAGE and transferred to nitrocellulose membrane using a transfer buffer (25 mM Tris-base, 192 mM glycine and 20% v/v methanol) at 86 mA overnight. The membrane was incubated in blocking buffer of 5% skim med milk/wash buffer (12.1 g/l Tris-base, 9.05 g/l NaCl, pH 7.4) with gentle shaking for 90 min at room temperature. The membrane was probed with 1:1000 solution of the CS3 polyclonal antiserum for 1h and then incubated with 1:1000 dilution of horseradish peroxidase-conjugated anti rabbit immunoglobulin (Tebsan, Iran) as secondary antibody and visualized using 4-chloro-1-haphtol with hydrogen peroxidase as the enzyme substrate.

**Immunofluorescence microscopy:** Bacteria from over night culture on CFA agar were gently resuspended in PBS. Cells were fixed on microscope slides, covered by 50 µl of the CS3 polyclonal antiserum as primary antibody diluted (1:1000) in PBS for 1h at room temperature. After washing five times with PBS solution, slides were incubated for 1 h with secondary antibody; Swine-anti Rabbit IgG fluorescein isothiocyanate (FITC) conjugated (DAKO, Denmark) at a dilution of 1:20. Cells were washed five times with PBS to remove unbound secondary antibody and observed by fluorescent microscope (Olympus).

**Heavy metal removal:** Heavy metal removal was tested using atomic absorption (GBC, M932 plus). Cultured cells on the CFA agar plates were collected and resuspended in 10 ml of LB. Several aliquots of the resuspended cells were precipitated at 15000×g for 30 min and the wet and dry weight (drying the biomass was carried out at 105ºC to constant weight) of the pellet was measured by a precise balance (Mettler, AT261).

The cells (100 mg wet weight) were resuspended in 30 ml of LB containing $Cd^{2+}$ [Cd $(NO_3)_2$. $5H_2O$], $Pb^{2+}$ [Pb $(NO_3)_2$] or $Ni^{2+}$ ($NiSO_4$) to final concentration of 20 µM of every metal. Samples were incubated

with gentle shaking for 2h at 37ºC. The cells were removed by centrifugation (6000×g, 5 min) and acetone was added to the supernatant at a final concentration of 80% (v/v) and incubated on ice for 15 min. The precipitated proteins were removed by centrifugation at 15000×g for 30 min (Pazirandeh *et al*., 1998). The supernatant was analyzed for heavy metal content by atomic absorption.

## RESULTS

**Construction of hybrid pili:** Determination of exposed area and secondary structure of CstH protein have been studied previously (Saffar and Yakhchali, 2005). Accordingly, the site between amino acids 65-66 of mature CstH was considered for insertion of the Cys-rich peptide. DNA fragment encoding N-terminal (amino acids 1-65) of CstH and cysteine rich peptide was amplified by PCR using M13 forward and B-Metal primers. The oligonucleotide B-Metal was designed in such a way that would allow correct insertion of the Cys-rich peptide into the CstH protein. Unique *Sac*I restriction site was introduced into amplified DNA for screening of the recombinant plasmids. *Pfu* DNA polymerase with proofreading property was used for amplification of the gene. The PCR product was purified and digested with *Bam*HI/*Xba*I enzymes and replaced with the corresponding fragment of the *cstH* gene in pPM4556 plasmid previously digested with the same restriction enzymes to produce plasmid pYS70. Recombinant plasmids were screened with *Sac*I restriction enzyme. Sequencing of the recombinant plasmid confirmed the fidelity of the hybrid gene. pPM4567 plasmid containing assembly cassette of the *cst* operon and pYS70 were digested with *Aoc*I /*Bam*HI restriction enzymes. The *cst* assembly cassette was cloned upstream of the hybrid gene to make hybrid operon. The resulting plasmid, pYS74, was used to transform *E. coli* cells to produce the recombinant pili.

**Expression and display of hybrid pili:** Expression of hybrid pili on the bacterial surface was investigated by Western blot analysis and immunofluorescence microscopy using polyclonal antibody against CS3 pili. Bacteria harboring pYS74 were cultured on CFA medium. Cells were harvested in PBS and pili was prepared. The hybrid pili was blotted on the nitrocellulose membrane and treated with polyclonal antibody against CS3. Expression of the hybrid pili was monitored in comparison with the negative (bacteria without CS3 operon) and positive (*E. coli* harboring pPM484 containing *CS3* operon) controls. Figure 3 shows strong reaction of the protein with anti-CS3 indicating that the hybrid pili was expressed and displayed on the surface of the *E. coli*.

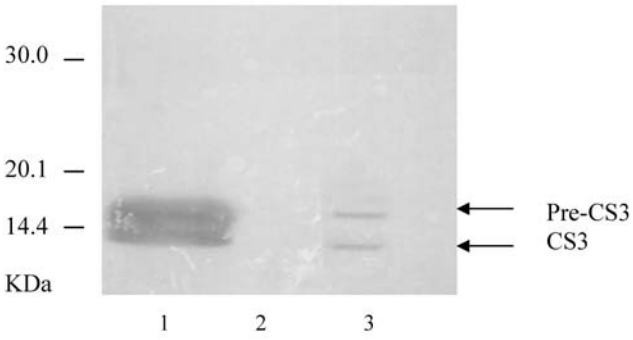


**Figure 3**. Western blot analysis of the *E. coli* expressing CS3 hybrid pili using CS3 polyclonal antibody. Lane, 1 positive control (*E. coli* cells expressing native pili); Lane 2, negative control (*E. coli* k12 TG1 host strain); Lane 3, pYS74 (*E. coli* expressing CS3 hybrid pili). The position of the precursor (pre-CS3) and mature forms of the CS3 pili are indicated (Jalajakumari *et al*., 1989).

Display of the Cys-rich peptide on the cell surface could be more directly confirmed by immunofluorescence microscopy. As shown in Figure 4, the *E. coli* cells expressing hybrid pili became fluorescent due to binding of anti-CS3 antibody followed by binding of FITC-conjugated secondary antibody, indicating that the Cys-rich peptide was successfully displayed on the cell surface (Fig. 4A). *E. coli* cells without pili (*E. coli* K12 TG1) were not fluorescent (Fig. 4B).

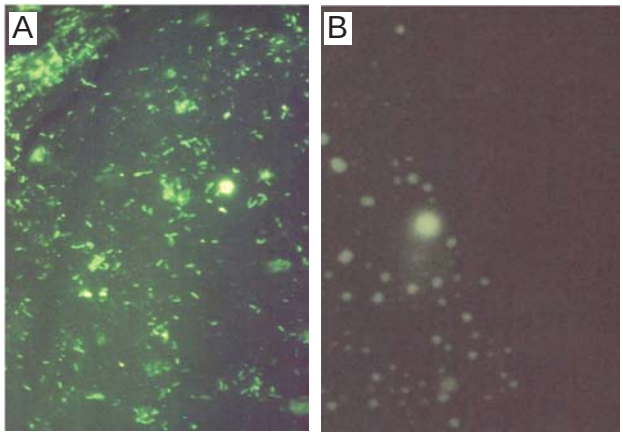


**Figure 4.** Immunofluorescence detection of hybrid CS3 pili. (A) Bacteria harboring plasmid pYS74 encoding hybrid CS3 pili, (B) Negative control cells (*E. coli* k12 TG1 host strain ). Cells were incubated with CS3 polyclonal antibody followed by probing with Swine-anti Rabbit IgG conjugated FITC and visualized with the Immunofluorescence microscope.

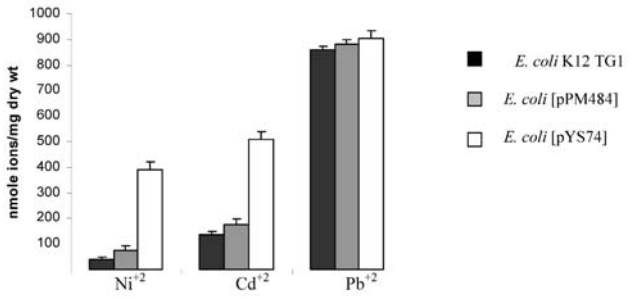


**Figure 5.** Bioaccumulation of heavy metal ions by *E. coli* pYS74 expressing hybrid CS3 pili and control cells (*E. coli* [pPM484] and *E. coli* K12 TG1). *E. coli* [pYS74] adsorbed 3-fold $Cd^{2+}$ and 10-fold $Ni^{2+}$ more than the *E. coli* [pPM484] (expressing native CS3 pili)**.** 100 mg wet weight of cells were resuspended in LB containing 20 μM of each metal ion. The metal content of the culture media was determined by atomic absorption after 2h of incubation. The bars represent the mean value of three independent experiments.

**Metal binding properties of *E. coli* displaying hybrid CS3 pili:** Bioaccumulation of bivalent metal ion experiments was carried out in the single metal ($Cd^{2+}$, $Pb^{2+}$ or $Ni^{2+}$) system. Insertion of the Cys–rich peptide into the CS3 pili led to the increase in removal of the $Cd^{2+}$ and $Ni^{2+}$ from media supplemented with 120 μM of $Cd^{2+}$ or $Ni^{2+}$. Metal binding properties of *E. coli* displaying hybrid pili were compared to the cells expressing native pili and cells without pili. Bacteria displaying hybrid pili accumulated $Ni^{2+}$ and $Cd^{2+}$ with efficiency higher than $Pb^{2+}$, compared to control cells. *E. coli* [pYS74] (expressing hybrid CS3 pili), could absorb 392.5, 510 and 905 nmol of $Ni^{2+}$ ,$Cd^{2+}$ and $Pb^{2+}$ per mg (dry weight) of cells, respectively (Fig. 5). Therefore, *E. coli* [pYS74] adsorbed 3-fold $Cd^{2+}$ more than the *E. coli* [pPM484] (expressing native CS3 pili) and *E. coli* K12 TG1 host strain**.** About ten- and five fold increase in bioaccumulation of the $Ni^{2+}$ was occurred in the cells expressing hybrid CS3 pili in compare to the *E. coli* cells producing native pili and host strain, respectively (Fig. 5). A slight increase in bioaccumulation of $Pb^{2+}$ was observed with cells expressing hybrid CS3 pili which were not significant in compare to the control cells (*E. coli* [pPM484] and TG1 strain) (Fig. 5). The surface display of cysteine rich peptide did not enhance the bioaccumulation of $Pb^{2+}$ in contrast with the accumulation of $Cd^{2+}$ and $Ni^{2+}$. This could be due to adsorption of $Pb^{2+}$ to the surface components of the cell but not to the peptide displayed on the cell surface.

## DISCUSSION

Widespread pollution by heavy metals has undesirable consequences for human health and environment (Sousa *et al*., 1998). Conventional methods for remediation of contaminated sites such as precipitation-filtration, ion-exchange, oxidation-reduction and membrane separation often fail to decrease the heavy metal contaminants to acceptable levels. Therefore, there exists a growing need for alternative methods capable of removing heavy-metal contaminants. Higher organisms such as plants and animals generally respond to heavy-metal challenge by production of cysteine-rich peptides such as metallothioneins and phytochelatins which bind metal ions (Wernerus and Stahl, 2004)**.** Metal binding peptides such as histidine-rich peptides have been used to construct bacteria with improved metalloadsorption characteristics using bacterial display approach (Kotrba *et al*., 1999; Samuelson *et al*., 2000; Sousa *et al*., 1996; Xu and Lee, 1999; Bae *et al*, 2000). Display of 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.

Fimbriae are adhesive bacterial surface structures and assisted display of heteologous peptides (Klemm and Schembri, 2000). The CS3 fimbriae of *E. coli* has been reported to tolerate the genetic insertion of heterologous peptides in position between amino acids 65-67 and 99-100 of mature CS3 (Yakhchali and Manning, 1997; Hosseini, 2003). The CstH peptide (major subunit of pili) consists of 144 amino acids with no cysteine residues in its sequence and can be considered as a candidate for insertion of cysteine rich peptides without interaction between the insert and carrier protein which affect its folding. Hence, the CS3 pili was used to display a cysteine-rich metal binding peptide for bioadsorption of heavy metals from contaminated effluents.

Different synthetic peptides such as cysteine and histidine rich peptides have been designed and used for construction of heavy metal bioaccumulating bacteria (Kotrba *et al*., 1999; Sousa *et al*., 1998; Wernerus and Stahl, 2004). Metal binding peptide Gly-Cys-Gly-Cys-Pro- Cys- Gly- Cys- Gly has been genetically engineered into LamB protein and expressed in *E. coli* (Kotbra *et al*., 1999). The genetic insertion of MBP sequence into CstH-65 resulted in CstH-MBP hybrid protein and retained its physiological properties as a pili located on the surface of the *E. coli* indicating maintenance of its overall folding pattern. Bacteria expressing hybrid LamB protein containing this metal binding peptide showed 1.8-fold increase in $Cd^{2+}$ binding capacity (Kotrba *et al*., 1999), whereas the cells expressing hybrid CS3::MBP pili showed 3-fold increase in $Cd^{2+}$ binding capacity. Recently the same results were obtained for bioaccumulation of the $Ni^{2+}$

(Wernerus and Stahl, 2004). This results may indicate that the CS3 pili is a more suitable display system for Cys-rich peptide for bioaccumulation of $Cd^{2+}$ and $Ni^{2+}$ and may be for other heavy metals.

*E. coli* cells have some heavy metal adsorption ability and the metal binding ability of *E. coli* cell wall has been studied (Ferris and Beveridge, 1985). The acidic groups of the exposed (hydrophilic) polypeptides and carboxyl and phosphoryl groups of lipopolysaccharides participate in metal binding. The peptidoglycan (PG) layer of the *E. coli* binds metal ions via the carboxyl and the hydroxyl groups of the glycan backbone (Kotrba *et al*., 1999). It seems that adsorption of $Pb^{2+}$ is attributed to the surface components of the cell but not to the Cys-rich peptide displayed on the cell surface.

The increased $Ni^{2+}$ and $Cd^{2+}$ binding capacity of the manipulated *E. coli* led to evaluate the influence of specific genetic insertions of Cys-rich metal binding peptide in metal removal.

We have demonstrated possibility of displaying cysteine rich peptide in a functional form on the surface of *E. coli* using CS3 pili for the generation of metal binding bioadsorbent to be used in environmental applications.

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