Surface display of metal fixation motif of bacterial P1-ATPases specifically promotes biosorption of Pb$^{2+}$ by *Saccharomyces cerevisiae*

Running title: Yeasts engineered for enhanced biosorption of lead

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Abstract

Biosorption of metal ions may take place by different passive metal-sequestering processes such as ion-exchange, complexation, physical entrapment, inorganic microprecipitation or their combination. To improve the biosorption capacity of the potential yeast biosorbent, short metal-binding NP peptides (harboring the CXXEE metal fixation motif of the bacterial Pb\(^{2+}\)-transporting P1-ATPases) were efficiently displayed and covalently anchored to the cell wall of *Saccharomyces cerevisiae*. These were fusions to the carboxyl-terminal part of the sexual adhesion glycoprotein α-agglutinin (AGα1Cp). Compared to yeast cells displaying the anchoring domain only, those having a surface display of NP peptides multiplied their Pb\(^{2+}\) biosorption capacity from solutions containing 75 to 300 µM of the metal ion up to fivefold. The S-type Pb\(^{2+}\) biosorption isotherms, plus the presence of electron dense deposits (with an average size of 80 × 240 nm, observed by transmission electron microscopy) strongly suggested that the improved biosorption potential of NP-displaying cells is due to the onset of microprecipitation of Pb species on the modified cell wall. The power of improved capacity for Pb biosorption was also retained by the isolated cell walls containing NP peptides. Their Pb\(^{2+}\) biosorption property was insensitive to the presence of a 3-fold molar excess of either Cd\(^{2+}\) or Zn\(^{2+}\). These results suggest that the biosorption mechanism can be specifically upgraded with microprecipitation by the engineering of the biosorbent with an eligible metal-binding peptide.
INTRODUCTION

Once released, non-degradable heavy metal species tend to persist indefinitely in the environment, circulating in the ecosystem, and eventually accumulating through the food chain. It is well known that each metal has its tolerated limit, above which it becomes toxic or hazardous (8, 25). Looking at the increased public awareness (even in the developing countries) over the issue of environmental toxicity of heavy metals, wastewater treatment is of the utmost importance. While most of the remediation methods in current use rely on physico-chemical processes with man-made synthetic materials, the use of microorganisms and plants as low-cost and eco-friendly alternatives of high efficiency is gaining increasing attention. Consequently, various bioremediation concepts are being proposed (4, 18, 23, 29, 34, 38, 41). Among them, the biosorption of metal ions with different types of biomass as biosorbents has proven an ideal bioremediation technology for metal-containing effluents, and occupies a position of the “traditional” bioremediation approach with several attempts at its commercialization (38, 41).

Biosorption of metal ions is a metabolism-independent metal accumulation event at the cell wall by polysaccharides, associated molecules, and functional groups. It involves mainly the ion-exchange, chemisorption, adsorption, and, in some cases, also the inorganic microprecipitation of certain heavy metal species (29, 38, 41). In the search for strategies allowing for enhancements of the biosorption capacity for a specific metal ion, the anchoring of particular amino acid sequences to the microbial cell wall has proved to be a promising approach. Surface displays of metal-binding oligopeptides, metallothioneins (MTs), or metalloproteins with the capacity to form coordination centers for the metal ions has been shown to improve the natural metallosorption ability of cells of *Escherichia coli*, *Staphylococcus xylosus*, and *Staphylococcus carnosus*. This approach was successfully extended to other environmentally robust bacteria and yeasts (reviewed in ref. 30). For
example, the engineering of mouse MT on the cell surface of *Cupriavidus metallidurans* (formerly *Ralstonia eutropha* and *R. metallidurans*) strain CH34 (7, 29) resulted in the MTB strain, which showed markedly improved capacity to immobilize Cd$^{2+}$ in soil, and to protect plants from the biological toxicity of the heavy metal (36). However, the power of surface-display-enhanced biosorption of metal ions was demonstrated with intact living microbes; whereas, biosorbents for wastewater treatment should preferably be formulated from non-living biomass (29, 38, 41).

In the present paper we describe the engineering of the carboxyl-terminal part of the sexual adhesion glycoprotein α-agglutinin (AGα1Cp) to anchor the metal fixation motif (CXXEE of the bacterial P1-ATPases) onto the cell wall of *Saccharomyces cerevisiae*. In many bacteria, certain P1-ATPases act as heavy metal ion-specific efflux pumps protecting the cell interior from metal toxicity (27, 33). A characteristic feature of the metal-transporting P1-ATPases is one or more heavy metal binding sites at the cytosolic amino-terminal end. Specifically, the CXXEE motif of PbrT of *C. metallidurans* CH34 is expected to be involved in the fixation of intracellular Pb$^{2+}$ prior to its export (3, 21). We show that the display of CXXEE on the surface of *S. cerevisiae* aided the natural Pb$^{2+}$-biosorption mechanism, with attendant microprecipitation events. It resulted in a substantial increase in the amount of cell-surface bound Pb. The acquired property was specific for Pb$^{2+}$, and also remained effective with the isolated cell walls.
MATERIALS AND METHODS

Strains, plasmids, media, and general procedures. The *S. cerevisiae* strain W303 (*MATa leu-2-3/112 ura-3-1 trp-1-1 his-3-11/15 ade-2-1 can-1-100 mal-10 GAL SUC2*) harboring constructs (Fig. 1), based on the centromeric expression vector p1V5-AG (37), was used for the surface display of fusions to AGα1Cp. The W303 strain was aerobically grown with shaking (200 rpm, 25-mm orbit) in YPD medium (1% [w/v] Difco yeast extract, 2% [w/v] Difco Bacto-Peptone, 2% [w/v] glucose, 0.003% [w/v] adenine hemisulfate) at 30°C. The surface-engineered cells harboring p1V5-AG variants were cultured in *URA*+ selective dextrose (SD) medium (0.7% [w/v] Difco yeast nitrogen base, 2% [w/v] glucose, 0.005% adenine hemisulfate, plus L-histidine, L-tryptophan and L-leucine each at concentrations of 0.003% [w/v]). Yeast cells from cultures at early stationary phase (optical density at 590 nm, OD<sub>590</sub> of 3 to 3.5) obtained after 20h of growth in YPD medium, inoculated with 5% of saturated culture in the same medium, were used in all experiments. *E. coli* TG1 (*supE hsdΔ5 thi Δ(lac-proAB) F'*traD36 proAB<sup>+</sup> lacI<sup>q</sup> lacZΔM15*), used to multiply the plasmids, was grown in Luria broth (31) with 150 µg of ampicillin ml<sup>-1</sup>. The DNA manipulations were performed according to standard protocols (31). The lithium acetate method (10) was used to transform host *S. cerevisiae*. The identity of *S. cerevisiae* transformants was verified by sequencing of the plasmid DNA, extracted using a Zymoprep™ kit (Zymo Research Corp., USA).

Genetic fusions for yeast surface display. Two complementary oligonucleotides (5’-GATCCCTAACTATCTCCAACATGGACTGTCCAACTGAAGAAGCTTTGATCAGA-3’ was the plus strand), used to construct fusions of NP peptides (Fig. 1) to the N-terminus of V5-AGα1Cp, were designed to produce a double
stranded NP fragment flanked at the 5'- and 3'-ends by BamHI and BglII cohesive termini, respectively. This DNA fragment was inserted into the unique BamHI site between the MFα1 and V5 sequences of p1V5-AG, giving rise to the plasmid p1AG-NP1 (Fig. 1). Only one BamHI site (at the 5’ end of NP) was then reconstituted within the resulting MFα1-NP-V5-AGα1C fusion. This allowed for successive insertions of the second and the third NP sequences in tandem, resulting in plasmids p1AG-NP2 and p1AG-NP3, respectively (Fig. 1).

**Immunofluorescence microscopy and transmission electron microscopy.**

Immunofluorescence microscopy was performed at room temperature, without the fixation of yeast cells. Approximately 10⁶ cells from early stationary cultures were washed twice with TBS (20 mM Tris-Cl pH 7.4, 150 mM NaCl, 3 mM KCl), incubated for 1 h in 200 µl TBS with 1% (w/v) bovine serum albumin. Next, the anti-V5-FITC monoclonal antibody (Invitrogen Corp., USA) was diluted 1:300 in the same buffer and was applied for 1 h. Cells were washed 3 times with 500 µl of TBS prior to microscopy. Fluorescence was detected using an Olympus IX81 microscope, equipped with a U-MWIBA/GFP filter (excitation 460-490 nm; emission 510-550 nm). The images were recorded using a model DP71 cooled digital camera and the CellB software (Olympus Corp., Japan).

Transmission electron microscopy (TEM) of unstained cells attached to the carbon-coated grids was conducted using a model JEOL JEM-1010, at magnifications ranging from ×10,000 to ×400,000 at 80 kV.

**Isolation and analysis of cell walls.** Cells of *S. cerevisiae*, grown to the early stationary phase in SD medium, were harvested by centrifugation for 5 min at 4,000 g and 25°C, and then washed with ice-cold isolation buffer (10 mM Tris-HCl, pH 7.8, 1mM PMSF). The
cells were resuspended at a density of 0.25 g (wet wt.) per 1 ml of the isolation buffer. Subsequently, 0.5 g of glass beads (0.5 mm diameter) were added for cell disruption in a Mini-Beadbeater device (BioSpec Products Inc., USA). This was conducted for 3 min at the maximum speed and 0°C. The glass beads were separated from the disintegrated cells by gravity. The supernatant and three successive washes of the beads with 1 ml of isolation buffer were pooled, and the cell walls were recovered by centrifugation for 5 min at 1,000 g and 4°C.

Cell wall proteins were fractionated as described elsewhere (32, 37). Briefly, the cell walls obtained from 1 g of cells (wet wt.) were washed 3 times with 10 ml of ice-cold 1 mM PMSF. Extraction of the cell walls was done with hot 2% SDS solution under reducing conditions; and then subsequent incubation with laminarinase (Sigma-Aldrich Corp., USA). This was followed by treatment of the released cell wall proteins with endoglycosidase H (Sigma-Aldrich Corp., USA). The extracted proteins were resolved by using 8% (w/v) acrylamide sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, and then electroblotted on nitrocellulose membranes. These were then blocked with 10% skim milk in TBS at 4°C for 16 h. The monoclonal anti-V5 antibody conjugated with horse radish peroxidase (anti-V5-HRP; Invitrogen Corp., USA). This was applied at a 1:5000 dilution in TBST (TBS with 0.1% Tween 20) with 2% skim milk for 2 h. Membranes were washed with TBST, and developed using a West Femto detection kit (Pierce Biotechnology, Inc., USA). The signal was scanned using a LAS 1000 luminescent image analyzer (Fuji Photo Film Co., Japan).

The nitrogen content of isolated cell walls was determined in triplicate in 2 mg of freeze-dried material by using an Elementar vario EL III analyzer (Elementar Americas, Inc., USA); 4-amino-bezenesulfonic acid was used as a standard.
Single- and two-metal biosorption assays in batch format. Fresh cells, harvested from *S. cerevisiae* cultures at an early stationary phase by centrifugation (4,000 g, 5 min, 25°C), or the isolated cell walls (see above) were washed twice with 25 mM MES (pH 5.5). The cells were resuspended to a final density of approximately 1.5 mg of dry cell wt ml⁻¹ (OD₅₉₀ of 2.5), and then with the cell wall material to a corresponding density of 0.45 mg of dry wt ml⁻¹ of the same buffer. Metal binding at pH 3 and 4 was performed in 25 mM MES/acetate, at pH 5 in 25 mM MES, and at pH 6 and 7 in 25 mM HEPES. The desired metal ions were added as Pb(NO₃)₂, Cd(NO₃)₂, and ZnCl₂ (Sigma-Aldrich Corp., USA) to the stated final concentrations. No precipitation of heavy metals in the test solutions was detected prior to the addition of the biosorbent materials. Following agitation at 130 rpm and 25°C for the stated period of time, the biosorbent materials were separated by centrifugation for 2 min at 4,000 g and 25°C. The metal content in the supernatant was determined by atomic absorption spectrometry (AAS; model Spectr AA300, Varian, Inc., USA). The separated biosorbent was washed with 25 mM HEPES (pH 6.5), and its metal content was determined by AAS after digestion with 70% nitric acid overnight under atmospheric pressure and at room temperature. Alternatively, washed cells were incubated for 15 min with 1/10 of the sorption solution volume of 10 mM EDTA (pH 7.0), in order to remove the surface-bound metal. The cells were then pelleted and treated as described above. Dry weights of the biosorbent were determined by drying the batch biosorption assay aliquots at 80°C.
RESULTS

Expression and surface display of NP peptide fusions to AGα1Cp. The NP peptide, harboring a putative metal fixation motif CXXEE was designed such that the conserved sequence MDCPTEELIR, along with the flanking residues, should mimic corresponding secondary structures within the P1-ATPases PbrT and CadA (Fig. 1). The secondary structure patterns were predicted using the hierarchical neural network prediction method (6) (not shown). To display up to three tandem NP peptide sequences on the cell wall of S. cerevisiae W303, the DNA fragments encoding NP were successively engineered to the MFα1-V5-AGα1C gene of the centromeric expression vector p1V5-AG (Fig. 1). This p416GPD-based (22) vector was obtained by fusion of the sequence encoding the leader peptide of α-factor precursor (MFα1) for surface targeting, V5 epitope tag for immunochemical detection, and the C-terminal anchoring domain AGα1Cp of α-agglutinin (37). The expression vectors for the surface display of one, two, and three NP peptides were named p1AG-NP1, p1AG-NP2, and p1AG-NP3, respectively (Fig. 1). We previously noted that use of centromeric constructions allows for uniform expression of fusions to AGα1Cp in all culture cells (37). When analogous 2µ vectors based on p426GPD (22) were used, corresponding fusions were displayed by only 20 to 75% of the culture cells (unpublished). A surface-display efficiency of 25.1% with a 2µ-based vector was also observed by Kuroda et al. (13), who attributed this phenomenon to mitotic instability of the vector.

Immunofluorescence analysis showed that virtually all cells of S. cerevisiae expressing MFα1-NPn-V5-AGα1C genes (n represents the number of NP repeats) labeled brightly fluorescent with the anti-V5-FITC antibody; confirming the constitutive expression and surface display of the corresponding proteins (Fig. 2A). Expression had no
adverse effect on culture growth, as determined in $URA^{+}$ selective SD medium, by comparing with the transformant harboring p416GPD.

To investigate the covalent attachment of NPn-V5-AGα1Cp to the cell surface, the cell wall proteins were fractionated and examined by immunoblotting. The non-covalently bound proteins were released from isolated cell wall materials by hot SDS treatment, and the covalently bound proteins were subsequently liberated by hydrolyzing the cell walls with endo-1,3(4)-β-glucanase laminarinase. The released proteins were then treated with N-acetylglucosaminidase H (EndoH) to reduce size heterogeneity, due to their N-linked polysaccharides. As shown in Fig. 2B, the substantial portion of NPn-V5-AGα1Cp and V5-AGα1Cp fusions was found in the EndoH-treated laminarinase extracts; thereby confirming the covalent modification of the cell wall glucan. The immunoblot further revealed that NP peptide extensions had no negative effect on the anchoring of V5-AGα1Cp variants to the cell wall. The 100 kDa band corresponding to the unbound V5-AGα1Cp variants, entrapped in the glucan network, was detected in all SDS extracts (Fig. 2B). A significant increase in the apparent molecular weights of the extracted fusion proteins, could be attributed to glycosylations of V5-AGα1Cp variants with predicted apoprotein sizes of 43.1 to 35.3 kDa. Such modifications involve O-glycosylations at abundant Ser and Thr residues, remnant of the cell wall β(1→6)-glucan and potential EndoH-resistant N-glycosylations (17, 32).

To estimate the number of displayed V5-AGα1Cp fusions, we determined the nitrogen content of cell walls by elemental analysis. While cell walls of wild-type S. cerevisiae W303 contained 2.41 ± 0.02 % of N, its content was found to be increased to 3.40 ± 0.03 % in those walls containing V5-AGα1Cp, and to 3.32 ± 0.02 % in those walls with NP1-V5-AGα1Cp or NP3-V5-AGα1Cp. Assuming that the increase in N content comes from the proteinaceous part of V5-AGα1Cp variants, the number of protein
molecules per cell was calculated as $4.1 \times 10^6$ for V5-AG\(\alpha\)1Cp, and $3.8 \times 10^6$ for NPn-V5-AG\(\alpha\)1Cp. Similar surface density ($1.6 \times 10^6$ copies per cell) was reported as natural for the protein CWP2 of \textit{S. cerevisiae} (9). However, other changes in cell wall composition due to the presence of V5-AG\(\alpha\)1Cp variants (\textit{e.g.}, increased chitin deposition to the cell wall) could not be excluded.

\textbf{Pb}^{2+} \textbf{binding properties of surface-engineered} \textit{S. cerevisiae}. The effect of the NP display on Cd\(^{2+}\), Zn\(^{2+}\), and Pb\(^{2+}\) binding capacity was initially tested using yeast cells expressing NP1-, NP2-, NP3-V5-AG\(\alpha\)1Cp, and V5-AG\(\alpha\)1Cp, grown to the early stationary phase. The cells (1.5 mg of cell dry weight ml\(^{-1}\)) were exposed to 150 \(\mu\)M of metal solutions (\textit{i.e.}, to a 400-fold excess of metal ions over the calculated number of displays in solution) at pH 5.5 and 7.0 for 4 h. While the display NP peptides had no effect on the sequestration of Cd\(^{2+}\) and Zn\(^{2+}\), it enhanced the capability of the cells to bind Pb\(^{2+}\). The amounts of accumulated Pb\(^{2+}\) were the same at pH 5.5 and 7.0, but reduced by 8\% at pH 5.0, and by more than 75\% at pH 4 and 3 (data not shown). The detailed biosorption isotherms were thus evaluated at pH 5.5, also to eliminate formation of PbOH\(^{+}\), which may occur at pH 7 at higher Pb concentrations. Compared to the control (displaying merely an anchoring domain), the cells displaying the NP peptides showed a sharp increase in their Pb\(^{2+}\)-binding capacity (Fig. 3A). This was most pronounced when initial concentrations of Pb\(^{2+}\) were 75 to 300 \(\mu\)M. The acquired property was essentially independent of the number of displayed NP peptide repeats per fusion protein (Fig. 3A shows data concerning the display of NP1 and NP3). The promoted metal sequestration capacity appeared solely to be due to a metabolism-independent biosorption of Pb\(^{2+}\) on the cell surface, as virtually all accumulated metal was released by washing with 10 mM EDTA from the cells pre-treated for 4 h with 50 and 150 \(\mu\)M Pb\(^{2+}\) (Fig. 3B).
To obtain insight into the kinetics of the Pb$^{2+}$ binding process, additional biosorption isotherms with the cells expressing NP1-, NP3-V5-AGα1Cp, and V5-AGα1Cp were evaluated under the same conditions over contact time periods of 45 min, 2 h, and 8 h. While only a slight contribution of the displayed NP peptides to biosorption of Pb$^{2+}$ occurred during 45 min (Fig. 4A), a gradual increase in Pb$^{2+}$ accumulation was observed with the cells displaying NP peptides after 2 h and 8 h of contact time (Figs. 3A and 4B, C). The most pronounced contribution of displayed NP peptides was observed at initial Pb$^{2+}$ concentrations of 100 to 150 µM. At these concentrations the biosorption capacity of the modified cells increased by factors of 3.5 to 5.2, compared to the V5-AGα1Cp-displaying control. The NP-displaying cells removed 90 and 95% of the metal after 4 and 8 h of contact time, respectively.

To investigate whether the yeast cell walls indeed gained the increased biosorption capacity due to the NP display, they were incubated in Pb$^{2+}$-containing solutions at pH 5.5 for 2 and 4 h. The used cell wall density corresponded to the cell wall mass proportion determined in the experiments with the intact cells. The cell walls enriched with NP peptides sequestered during 2 and 4 h of contact time up to 2.5 and 3-fold more Pb$^{2+}$, respectively, compared to the control walls containing V5-AGα1Cp protein alone (Fig. 5). The biosorption capacity of the modified walls was again independent of the number of NP repeats per fusion, and was highly effective at the initial Pb$^{2+}$ concentrations of 100 to 150 µM, when 90 to 95% of the metal was immobilized.

**Analysis of Pb$^{2+}$ deposition form.** A sharp increase in the amount of cell wall-associated metal which occurs after exceeding a certain threshold concentration of metal ions in solution often signalizes the precipitation of metal species on the surface of the biosorbent (1, 20). Thus, transmission electron microscopy (TEM) was employed to check for the
presence of precipitated Pb species within the cell walls (Fig. 6). While there was no precipitate detected in the controls (Fig. 6B and D), electron dense particles with an average size of 80 × 240 nm were observed on the cells displaying NP peptides, and treated with 150 µM Pb Pb²⁺ for 4 h (Fig. 6A and C).

Specificity of displayed NP peptide, and effect of competing ligands. As indicated above, we did not observe any contribution of NP peptides to the Cd²⁺ and Zn²⁺ binding capacity of S. cerevisiae cells incubated in the presence of 150 µM concentrations of the respective metal ion. This prompted us to investigate the specificity of NP display for the biosorption of Pb²⁺ in two metal systems. Therefore, the isolated cell walls were incubated with 100 µM Pb²⁺ in the presence of 300 µM Cd²⁺ or Zn²⁺. This experiment showed that none of the competing metal ions affected the biosorption of Pb²⁺ (Fig. 7). In contrast, the biosorption of Cd²⁺ and Zn²⁺ was reduced by 40 to 50%, compared with the biosorption data obtained with the single metal systems. The isolated cell walls containing NP peptides also showed a slightly improved capacity for biosorption of Zn²⁺ and Cd²⁺ from both the single and the double metal solutions (Fig. 7). The same metal binding properties were observed with the intact cells treated under identical conditions; except that the NP peptides did not contribute to biosorption of Zn²⁺ and Cd²⁺ (not shown).

To test whether competing metal chelating molecules may inhibit biosorption by modified cell walls, we studied the effects of some commonly known Pb chelators. Specifically, EDTA, citrate, and glutathione were added in biosorption experiments with 150 µM Pb²⁺. The effects of these competitors were generally independent of the presence of NP peptides. EDTA exerted complete inhibition when added at 150 µM (not shown). While glutathione showed only a slight inhibition, 5 mM citrate reduced the amount of biosorbed Pb by 75 to 87% (Fig. 7B).
DISCUSSION

The covalent attachment of NP peptides harboring the CXXEE metal fixation motif of the bacterial metal transporting P1-ATPases, on the surface of *S. cerevisiae* (Fig. 2), was expected to promote the biosorption of metal ions on the modified yeast cell wall. The rationale behind the choice of the CXXEE motif, rather than of the [designed] high-affinity metal-binding center, was its expected propensity to exchange the metal ion; an event which should occur during metal export by the transporter ATPase. It had been well documented, that displays of metal-binding peptides enhance biosorption of metal ions on the bacterial cell walls (reviewed in ref. 30). This effect was due to the capacity of the displayed peptides to bind metal ions, as well as to their competence to exchange the metal ion with the cell wall structures, which otherwise cannot react with the metal ion in solution (12, 35, 36). Display of the hexahistidine or yeast MT has also been reported to multiply the natural biosorption capacity of *S. cerevisiae* for Cd$^{2+}$ and Cu$^{2+}$ (14, 15, 16). However, the display of HP peptide GHHPHG increased the amount of adsorbed Zn$^{2+}$ by only 20% (37), which was consistent with the HP:Zn$^{2+}$ stoichiometry of 1:1 (with an apparent dissociation constant of 120 nM). Such properties of the HP peptide indicate that the display of a high affinity peptide does not support “funneling” of the metal ion to the natural metal-binding sites of the *S. cerevisiae* cell wall.

The CXXEE motifs naturally localize at the cytosolic amino-terminal end of the PbrA transporter of the Pb$^{2+}$-specific resistance system (3); and also of the Pb$^{2+}$ and Cd$^{2+}$ transporting CadA of *C. metallidurans* CH34 (21). It occurs within the conserved sequence MDCPTEEALIR (Fig. 1). When this sequence was displayed on the surface of *S. cerevisiae* as a part of the NP peptide, it markedly promoted the biosorption of Pb$^{2+}$ (Figs. 3A and 4). An improved capacity to sequester Pb$^{2+}$ from solution was also retained by the isolated NP-containing cell walls of *S. cerevisiae* (Fig. 5). It confirmed that the
functionality of NP peptide is independent of intracellular metabolism, and that the 
accumulation of Pb$^{2+}$ can be solely attributed to the biosorption process. Here, it should be 
noted that we had previously observed a substantial reduction in the contribution of 
displayed peptides to the biosorption capacities of engineered cell walls of *E. coli* and *S.
cerevisiae*, compared to those of intact cells (12, 37); demonstrating that the fully native 
cell wall architecture played a critical role in these particular cases.

The cell wall of *S. cerevisiae* is a multilaminate, microfibrillar structure composed 
mainly of β(1→3)-D-glucan, β(1→6)-D-glucan, chitin, (phospho)mannoproteins, and a 
minor proportion of lipids and pigments (17). Functional groups such as carboxylate, 
phosphate, hydroxyl, amine, sulfhydryl, and imidazole contained in these biomolecules 
participate in sequestering of the metal ions via bonds of either an ionic or covalent nature 
(2, 40, 41). The biosorption mechanism of heavy metal ions involving the ion-exchange 
and complexation is characterized by L-type biosorption isotherms with initially higher 
slope that decreases with increasing equilibrium metal concentration. (1, 11, 38). The L-
type Pb$^{2+}$-biosorption isotherms were obtained for *S. cerevisiae* producing the anchoring 
domain only (Figs. 3A, 4 and 5). The biosorption isotherms observed for the cell walls 
gineered with NP peptides (Figs. 3A, 4B and C, and 5) can be regarded as the S-type. 
The S-type isotherms are generally characterized by a lower initial slope, which sharply 
increases as the equilibrium concentration increases (1, 11, 38). Such biosorption isotherms 
are indicative of the inorganic microprecipitation of metals on biosorbent, initiated by 
heterogeneous nucleation (11, 20). Nucleation and microprecipitation of metal hydroxides 
or phosphates may result from locally increased concentration of surface-bound metal ion 
and increased pH or presence of biogenic phosphates (11, 20, 24, 38). Given the S-shape of 
biosorption isotherms (Fig. 3A, 4B, C, and 5), as well as the presence of electron dense 
particles (Fig. 6A and C), we deduce that the microprecipitation of Pb species is
responsible for the enhanced biosorption property of the NP-peptides-containing cell walls. However, the chemical form of the microprecipitate remains to be investigated. A microprecipitation phenomenon had only been described in *S. cerevisiae* for uranium biosorption (39). The microprecipitation of Pb on a natural biomass was observed with the alga *Sargassum vulgaris*, in which the metallic Pb$^0$ became deposited (26). Microprecipitation of Pb, presumably as hydroxides and phosphates, was observed with *Rhizopus arrhizus* (24). The metal-based titration of the *R. arrhizus* biomass at pH 5 allowed Naja *et al.* (24) to show that the formation of insoluble Pb species occurred after the initial binding of Pb$^{2+}$ via ion-exchange at a lower saturation of the biomass with the metal. Microprecipitation became evident at a higher saturation, when the equilibrium Pb$^{2+}$ concentrations in solution exceeded micromolar levels.

It appears reasonable to assume that the contribution of the NP peptide involves a gradual increase in the local Pb$^{2+}$ concentration to a certain level that triggers the microprecipitation. Intriguingly, the overall biosorption capacity of modified cells was essentially independent of the number of NP peptide repeats displayed per fusion protein (Figs. 3A, 4, and 5). In our model we assume that the single CXXEE motif has sufficient power to promote formation of the nucleation sites for microprecipitation. However, it remains unclear why the effect of the display of NP peptides is lost in the intact cells when the initial Pb$^{2+}$ concentrations exceed 300 µM (Figs. 3A and 4); whereas, the isolated NP-engineered cell walls remained effective even at 500 µM Pb$^{2+}$ (Fig. 5).

A number of biosorption studies have shown that the Pb$^{2+}$ biosorption capacity of the natural biomass of *S. cerevisiae* exceeds that for Cd$^{2+}$ and Zn$^{2+}$ by a factor of 4 (5, 40), making this yeast a suitable candidate for a biosorbent to be utilized in the Pb$^{2+}$ remediation process. Current efforts in biosorption research are mainly dedicated to algal biosorbents, which show both a high efficiency at low metal concentrations and high
biosorption capacities (38, 41). The best performing are biosorbents made of brown marine
algae, which show average maximum biosorption capacities of 1240 nmol of Pb mg⁻¹;
however, at high equilibrium metal concentrations of about 1 mM (40). The maximum
biosorption capacity of NP-containing cell walls of *S. cerevisiae* was apparently 300 nmol
of Pb mg⁻¹ (Fig. 5). Based upon data obtained by Romero *et al.* (28) with representative
algal biosorbents and under similar experimental conditions (biosorbent density of 0.5 mg
ml⁻¹, pH 5.0), the biosorption capacity at equilibrium concentration of 350 µM Pb²⁺ (given
in parentheses as nmol mg⁻¹) decreased in the following order: *Fucus spiralis* (885, brown
algae) > *Ascophyllum nodosum* (747, brown algae) > *Chondrus crispus* (410, red algae) >
modified *S. cerevisiae* (291, NP1-containing walls) > *Codium vermilara* (267, green algae)
> *Spirogira insignis* (244, green algae) = *Asparagopsis armata* (235, red algae) > *S.
cerevisiae* (130, cell walls with AGα1Cp only). However, the contribution of NP peptides
was most pronounced at lower Pb²⁺ concentrations (Fig. 5). Indeed, the order of the
biosorption capacity at equilibrium of 9 µM Pb²⁺ changes in favor of NP-containing cell
walls: NP1-modified *S. cerevisiae* walls (193) = *F. spiralis* (189) > *S. insignis* (128) = *A.
nodosum* (125) > *C. vermilara* (52.1) > *S. cerevisiae* walls with AGα1Cp only (36.8) > *A.
armata* (23.4) > *C. crispus* (17.9).

In summary, we have shown that the Pb²⁺ biosorption capacity of *S. cerevisiae* can
be substantially improved by the surface display of the specific metal-binding peptide NP.
The contribution of NP peptides was not impaired by an excess of potentially competing
Cd²⁺ and Zn²⁺ ions (Fig. 7A). Moreover, virtually all the Pb can be recovered from the cell
surface by EDTA treatment (Fig. 3B), and the contribution of the NP peptides also
remained effective in the isolated cell walls. Such properties of modified cell walls could
be of practical importance, since the biosorbents are based on non-living biomass. The
feasibility of usage of a biosorption column packed with silica-immobilized cell walls of *S. cerevisiae* has already been demonstrated (19).

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**REFERENCES**


FIGURE LEGENDS

FIG. 1. The organization of expression vectors for the surface display of NP peptide fusions to V5-AGα1Cp. The vector p1AG-NP2 has the same organization but contains two NP repeats in tandem. The sequence of NP peptide is shown with its predicted secondary structure italicized above. The consensus CXXEE-containing sequences of PbrA (GenBank accession no. YP145622), and the chromosomally-encoded CadA (GenBank accession no. ABF11456) of C. metallidurans CH34 are shown boxed. GPDpr – constitutive glyceraldehyde-3-phosphate dehydrogenase promoter, CYC1ter – cytochrome-c oxidase terminator, c – random coil, e – extended strand, h – helix.

FIG. 2. Surface display of NP1 and NP3 fusions to V5-AGα1Cp expressed in S. cerevisiae W303. (A) The Normarski interference micrographs (upper panels) and matching immunofluorescence micrographs (bottom panels) of cells transformed with the indicated expression vectors (Fig. 1) and probed with the anti-V5-FITC antibody. (B) Immunochemical detection of indicated V5-AGα1Cp variants with the anti-V5-HRP antibody in the cell wall extracts from the S. cerevisiae W303 transformants. SDS - SDS extractable non-covalently bound proteins; EndoH - laminarinase extractable covalently anchored proteins treated with endoglycosidase H.

FIG. 3. Biosorption of Pb^{2+} by surface-engineered S. cerevisiae strains. (A) The metallosorption isotherms of cells displaying NP1-V5-AG1αCp (triangles); NP3-V5-AG1αCp (squares); and V5-AG1αCp alone (circles). Shown are the representative biosorption isotherms obtained in parallel after 4 h of contact time in the 1 to 750 µM Pb^{2+} solutions at pH 5.5 and 25°C. The inset graph shows initial part of the isotherm. The cell density was 1.5 mg of cell dry weight ml^{-1}. (B) The amount of Pb^{2+} recoverable from the
cells by EDTA treatment. Indicated transformants were preadsorbed with Pb\(^{2+}\) to the levels shown by the outside columns. The white inside columns show the amount of Pb removed from preadsorbed cells by treatment with 10 mM EDTA (pH 7.0). Data are mean values of three replicate experiments ± standard deviation of the mean.

**Fig. 4.** Kinetics of Pb\(^{2+}\) biosorption by surface-engineered *S. cerevisiae*. The representative biosorption isotherms obtained in parallel after: (A) 45 min, (B) 2 h and (C) 8 h of contact time in the 10 to 500\(\mu\)M Pb\(^{2+}\) solutions at pH 5.5 and 25°C are shown for cells displaying NP1-V5-AG1\(\alpha\)Cp (triangles); NP3-V5-AG1\(\alpha\)Cp (squares); and V5-AG1\(\alpha\)Cp alone (circles).

**Fig. 5.** Biosorption of Pb\(^{2+}\) by isolated surface-engineered cell walls of *S. cerevisiae*. The representative biosorption isotherms of the isolated cell walls displaying NP1-V5-AG1\(\alpha\)Cp (triangles); NP3-V5-AG1\(\alpha\)Cp (squares); and V5-AG1\(\alpha\)Cp alone (circles) are shown. Isotherms were obtained in parallel after contact times of 2 h (closed symbols) and 4 h (open symbols) in the 25 to 500\(\mu\)M Pb\(^{2+}\) solutions at pH 5.5 and 25°C. The inset graph shows initial part of the isotherm. The cell wall density was 0.45 mg of wall dry weight ml\(^{-1}\).

**Fig. 6.** Deposition of Pb microprecipitate at the NP-containing cell walls. (A, C) The cells producing NP3-V5-AG1\(\alpha\)Cp and preadsorbed in the 150\(\mu\)M Pb\(^{2+}\) solution, as described in Fig. 3A, and then directly inspected by TEM without fixation or staining. The electron-dense microprecipitate particles are indicated with arrows. (B) The cells producing V5-AG1\(\alpha\)Cp alone and treated as described in the legend to panel A. (D) The cells producing NP3-V5-AG1\(\alpha\)Cp incubated under the same conditions but in the absence of Pb\(^{2+}\).
FIG. 7. Specific contribution of NP peptide display for biosorption of Pb$^{2+}$ and the effect of competing ligands. Cell walls isolated from the indicated transformants were incubated with the metal ions or competing ligands at pH 5.5 and 25°C for a period of 2 h. (A) Selective biosorption of Pb$^{2+}$ in the presence of competing Cd$^{2+}$ and Zn$^{2+}$. Biosorption of metals was scored in single metal systems with 100µM Pb$^{2+}$ and/or 300µM Cd$^{2+}$ or Zn$^{2+}$, and in double metal systems with 100µM Pb$^{2+}$, plus 300µM of either Cd$^{2+}$ or Zn$^{2+}$. Data are mean values of at least three replicate experiments ± standard deviation of the mean. (B) Effect of Pb-chelators on biosorption. Cell walls were incubated with 150µM Pb$^{2+}$ and various concentrations of glutathione or sodium citrate. Data are the mean values of two replicate experiments (bars indicate their span).
(A) $Q_e$ (nmoles metal [mg dry wt$^{-1}]$)

- p1V5-AG
- p1AG-NP1
- p1AG-NP3

metal (in the presence of)

Pb, Pb(Cd), Cd(Pb), Pb(Zn), Zn(Pb), Cd, Zn

(B) $Q_e$ (nmoles Pb, [mg dry wt$^{-1}$])

p1V5-AG:  ; p1AG-NP3: 

glutathione (µM) 150 900 1500 5000

citrate (µM) 150 900 1500 5000