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Characterization and Comparison of Metal Accumulation in Two *Escherichia coli* Strains Expressing Either CopA or MntA, Heavy Metal-Transporting Bacterial P-Type Adenosine Triphosphatases

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Abstract

MntA from *Lactobacillus plantarum* and *copA* from *Enterococcus hirae* both encode membrane proteins that are members of the P-type family of adenosine triphosphatases (ATPases). Both transporters act as metal importers to take up nutritionally required substrates; MntA translocates Mn(II) and CopA translocates Cu(I). Both ATPases can also translocate secondary substrates, Cd(II) and Ag(I), respectively. Although functionally and sequentially similar, these ATPases differ in several key residues and in their membrane topologies. The bioaccumulation properties of these two proteins were examined by coexpressing the transporters with overexpressed metallothionein in *Escherichia coli* cells, a system that has previously shown high levels of substrate-specific uptake. Both strains exhibited rapid metal accumulation, both saturated at around 50 μ*M* metal, and both displayed temperature-sensitive uptake. However, the transporters responded differently when external conditions were varied; MntA displayed increased sensitivity to ionic strength, while CopA was more pH sensitive and more inhibited by chelating agents. The differences in accumulation are likely owing to structural differences in the transmembrane region of these two ATPases.

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Index Entries: Ion transport; P-type adenosine triphosphatase; copper; cadmium; genetic engineering; metallothionein; *lactobacillus*; *enterococcus*; *Escherichia coli*.

Introduction

In the modern industrial world, anthropogenic heavy metal pollution continues to be a problem. Although there are already many methods designed for heavy metal cleanup, they tend to be expensive, as well as nonspecific and sensitive to ambient conditions. Intracellular bioaccumulation with genetically engineered microorganisms has been considered one possible alternative to overcome current deficiencies *(1)*. In a laboratory environment, it is very easy to use genetic engineering to introduce specific, desired traits into a microorganism to produce a tool for bioremediation. This approach has already been used to construct cells for bioremediation of mercury and nickel *(2,3)*. The technique constructs cells that coexpress a membrane transport system and an intracellular sequestration system in the form of a glutathione *S*-transferase–metallothionein fusion protein (GST-MT). Such a combination allows for rapid accumulation of specific metal ions in a bacterial cell without toxic effects.

In the present study, two *Escherichia coli* strains were engineered to coexpress a membrane transporter and metallothionein. The transporters chosen were MntA from *Lactobacillus plantarum* and CopA from *Enterococcus hirae*. *L. plantarum* MntA is an Mn^{2+} importer, responsible for maintaining a high intracellular concentration of Mn^{2+}, which the organism utilizes to inactivate oxygen radicals *(4)*. CopA is a Cu^{+} importer and, along with the exporter CopB, is responsible for copper homeostasis in *E. hirae (5)*. Both adenosine triphosphatases (ATPases) also have secondary substrates; MntA imports Cd^{2+} and CopA imports Ag^{2+}, neither of which is a nutritionally required ion. The two proteins are in the same superfamily and share approx 25% sequence similarity, and several key regions, such as the ATP-binding site and the aspartyl kinase domains, are well conserved. On the other hand, the putative metal-binding regions are different, and the two proteins exhibit different membrane topology.

Both proteins are P-type ATPases. P-type ATPases form a large and broad family of membrane-associated transport pumps. These proteins have been classified into different subfamilies according to substrate specificity. Known substrates include protons, hard Lewis acids (K, Mg, Na), soft Lewis acids (Cu, Cd, Zn), and even phospholipids *(6)*. Widespread in nature, P-type ATPases are found in archaea, prokaryotes, and eukaryotes ranging from yeast to humans. P-type ATPases are very good candidates for use as a transporter in the GST-MT-engineered cells, beyond even bioremediation applicability. There are dozens of defined ATPase systems for a variety of metals to choose from, and since it is a large, related family, comparing the results of different transporters can yield some insight into the underlying mechanisms of the P-type family as a whole. In addition,

since P-type ATPase divergence is based on substrate specificity, and not the evolutionary lines of the organism, knowledge of the bacterial systems can be readily applied to eukaryotic ones. We conducted several experiments to study the applicability of these cells for bioremediation, as well as to compare the metal uptake characteristics of these similar yet different ATPases.

Materials and Methods

Strain and Plasmid Construction

To construct plasmid pKCOP, plasmid pY164 *(7)*, which contains the *E. hirae CopA* gene, was amplified by polymerase chain reaction (PCR) (five cycles) using primers CxxC-Up (5'tcacacaggTaCcTgaattcatggcaacga 3') and CopA-Dwn (5' ctgcagcccgggtctagagt cgacttatttg 3') and an Expand high-fidelity enzyme kit. The PCR product was then cloned into the pCR2.1-TOPO TA cloning vector, and plasmids with inserts were selected on the basis of blue/white screening and kanamycin resistance (60 µg/mL), giving plasmid pCRCOP. It was next digested with *Kpn*I and *Xba*I, and the resulting 2.2-kb fragment was ligated onto the corresponding site in plasmid pK187 *(8)*, giving plasmid pKCOP. To construct plasmid pKMNT, plasmid pZH3-5 *(9)*, which contains the *L. plantarum MntA* gene, was amplified by PCR (five cycles) using primers 3-5N-Fu-2 (5' aattcgGggAtCcag acatgatttgaagattt 3') and MntC-Lwr (5'tatcacCTGcaGtatgagcattgtaat catttagtc 3') and the Expand high-fidelity enzyme kit. The PCR product was then cloned into the pCR2.1-TOPO TA cloning vector, and plasmids with inserts were selected on the basis of blue/white screening and kanamycin resistance (60 µg/mL), giving plasmid pCRMNT. It was next digested with *Bam*HI and *Pst*I, and the resulting 2.3-kb fragment was ligated between the appropriate sites on pK187 *(8)*, to give pKMNT.

After ligation, all plasmid products were transformed into $CaCl_2$ chemically competent *E. coli* DH5α cells, and transformants were selected on the basis of the appropriate antibiotic resistance. Single colonies were grown up in Luria Bertani (LB), and plasmid DNA was purified and screened by restriction digests to ensure proper insertion and orientation of the inserts.

To construct strains expressing a membrane transporter and/or metallothionein fusion protein, pKMNT, pKCOP, or host vector pK187 was separately cotransformed with either pGPMT or host vector pGEX-3X *(10)* by heat shock of chemically competent *E. coli* JM109 cells *(11)*. Transformed cells were selected on LB plates on the basis of dual antibiotic resistance (60 µg/mL of kanamycin for pK derivatives and 100 µg/mL of ampicillin for pGEX derivatives). Single colonies were grown up in LB, and plasmid DNA was purified and screened by *Eco*RI digestion to ensure the presence of both plasmids.

Analysis of Protein Expression and Heavy Metal Sensitivity and Resistance

Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis and heavy metal resistance assays, to determine proper protein expression and function, were carried out as in ref. *3*. Both strains expressed the fusion protein at a high level and had the expected metal resistance.

Measurement of Copper and Cadmium Bioaccumulation

E. coli cells harboring the desired plasmids were grown in appropriate medium and induced with 1.0 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) as described previously. Cells containing the MntA transporter were grown in LB, while cells containing the CopA transporter were grown in M9 minimal medium +1% LB to minimize exposure to copper prior to accumulation. After induction, cells were harvested by centrifugation and resuspended in 10 m*M* phosphate buffer, pH 7.0. Depending on the plasmid, $CuSO_4$ or $CdCl_2$ was then added to the suspension, and the cells were incubated at 37°C with shaking.

To test the effects of temperature on bioaccumulation, cells were induced and resuspended as described previously and then incubated at either 4 or 20°C instead of 37°C. To determine the effect of ionic strength on either cadmium or copper uptake, induced cells were suspended in buffer containing sodium chloride (0–50 m*M*), magnesium chloride (0–10 m*M*), or calcium chloride (0–1 m*M*). For analysis of selectivity, cells were resuspended in buffer containing cadmium chloride, cobalt chloride, copper sulfate, manganese sulfate, nickel chloride, silver nitrate, or zinc sulfate (50 μ*M* for silver nitrate, 100 μ*M* for all others). To determine the effect of chelators, induced cells were suspended in buffer along with either 1 m*M* ethylene diamine tetraacetic acid (EDTA) or 1 m*M* sodium citrate. To study energy dependence, cells were incubated along with 1.0 m*M* potassium cyanide (KCN), 1.0 m*M* 2,4-dinitro phenol (DNP), or 0.4% glucose. For pH studies, cells were incubated in phosphate buffer altered to a pH ranging from 3.0 to 11.0. After incubation, cells were centrifuged, washed three times with cold phosphate buffer, frozen at –70°C, lyophilized for at least 8 h, and digested in 70% nitric acid at 50°C for 16–20 h. Samples were then diluted with H_2O to a final concentration of 1 mL and analyzed for metal content by flame atomic absorption spectrophotometry on a Perkin Elmer 2380 AAS.

Measurement of Cellular ATP

E. coli cells harboring the desired plasmids were grown in appropriate medium; induced with 1.0 m*M* IPTG; and incubated in phosphate buffer with glucose, KCN, or DNP as described previously. After 0, 15, or 30 min, 1 mL of cell solution was taken, spun down, and washed once with cold buffer. The cells were then resuspended in ATP extraction buffer (20 m*M* glycine; 50 m*M* Mg^{2+}; 4 m*M* EDTA, pH 7.4) to an optical density of 2.0. Intracellular ATP was extracted by boiling the cells for 1 min.

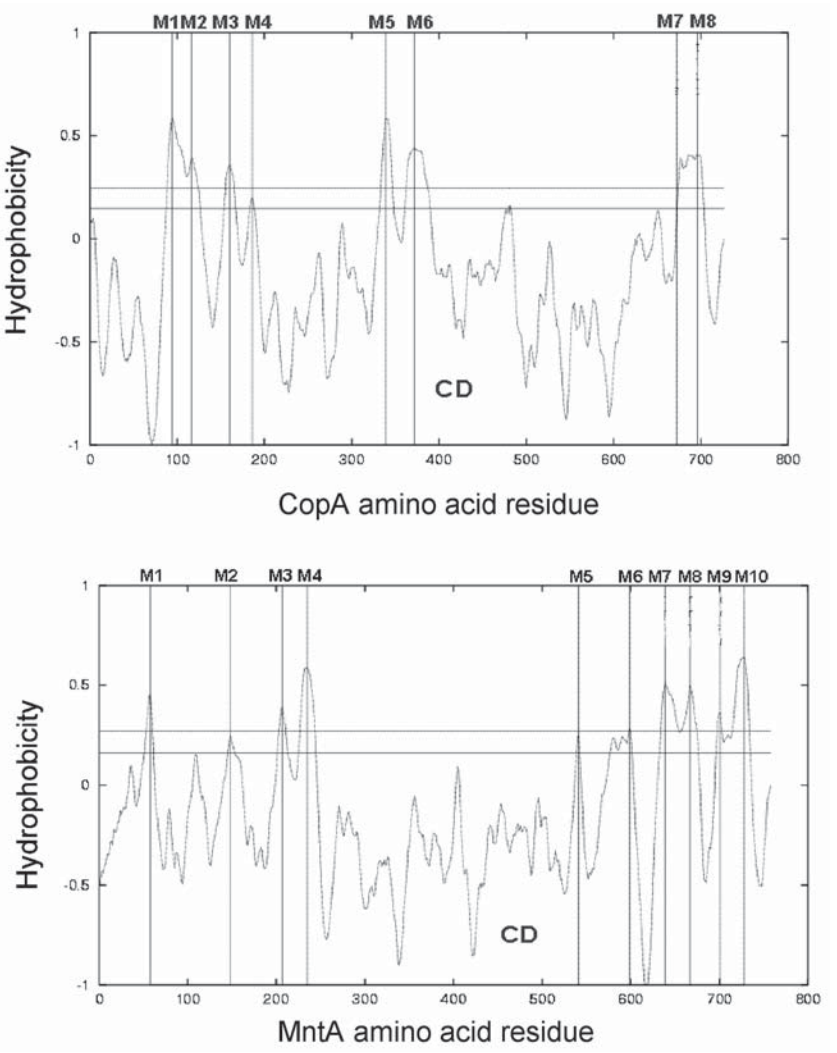

Fig. 1. Hydropathy plots of *E. hirae* CopA **(A)** and *L. plantarum* MntA **(B)**. Vertical lines indicate the location of putative transmembrane segments (M); the location of large cytoplasmic domain (CD), containing ATP and kinase-binding sites, is also indicated.

Ten microliters of cell solution was combined with 100 µL of luciferin/luciferase reagent (2 mg/mL; Sigma, St. Louis, MO), and luciferase activity (relative luminescence unit [RLU]) was measured on a luminometer. Values were compared to an ATP-standard curve.

Results and Discussion

Sequence and Structural Comparisons of MntA and CopA

The hydropathy profiles of the two ATPases are shown in Fig. 1; predictions using these plots suggest that CopA has eight putative transmembrane helices, like other members of the heavy metal subfamily, whereas

MntA has 10 transmembrane helices, similar to P-type ATPases that transport hard Lewis acids *(12)*. The MntA protein also lacks the distinct cysteine-containing amino-terminal domain that is found in other heavy metal P-type ATPases *(13)*. Another difference between these two proteins is the composition of the metal-binding region. Two cysteine pairs, one near the N-terminus, the other on transmembrane helix 6, are believed to be responsible for copper binding *(7)*. In these same regions, MntA contains several reactive oxygen-containing side chains, such as Asp, Asn, and Glu *(9)*.

Analysis of Copper and Cadmium Bioaccumulation in Engineered Cells

To determine the rate of metal bioaccumulation as well as the saturation point of the engineered cells, metal bioaccumulation assays were done varying incubation time, incubation temperature, and initial metal concentration (Fig. 2A–F). Although manganese is the biologic substrate, cadmium was chosen for MntA studies for three reasons: First, cadmium and copper have similar metallothionein binding affinity and coordination geometry *(14)*. Second, cadmium and copper are the two substrates that have higher affinity for their ATPases; this is interesting regarding MntA, which has a higher affinity for a metal ion of no biologic significance *(9)*. Third, cadmium is more toxic.

Cells that expressed both a transporter and a metallothionein had significantly higher levels of accumulation than cells expressing only metallothionein; there was a sixfold increase for MntA-expressing cells and a threefold increase for CopA cells at a concentration of 10 μ*M* metal. Part of the reason for the lower effect of the CopA transporter was the higher background accumulation, likely owing to various endogenous metal transporters *(15,16)*. Both engineered strains showed rapid initial uptake (>60% total metal accumulation within the first 10 min) as well as effective uptake at low metal concentrations (<5 μ*M*). The MntA transporter was more effective at low concentrations; at 1 μ*M*, MntA accumulated 8.56 μmol of Cd/g of dry wt, whereas CopA accumulated 4.74 μmol of Cu/g of dry wt. As the concentration increased, the accumulation levels became more comparable. The two strains had similar saturation levels (approx 50 μmol/g of dry wt), which is not surprising, considering that copper and cadmium have similar metallothionein binding geometry and affinity.

Accumulation was temperature sensitive, with reduced uptake at 20°C and almost no uptake at 4°C. Lowering the temperature would have many effects on membrane fluidity, enzyme activity, and aqueous diffusion *(17)*; thus, although these results do not necessarily prove the involvement of active transport in metal accumulation, they do support the possibility. The high accumulation observed at 20°C demonstrates that these cells can work in environmental conditions in which temperature may not be ideal.

A Michaelis-Menton plot of accumulation vs concentration illustrates the differences between these transporters (Fig. 3). The K_m values are very similar, but there is a noticeable difference in the V_{max} of these proteins.

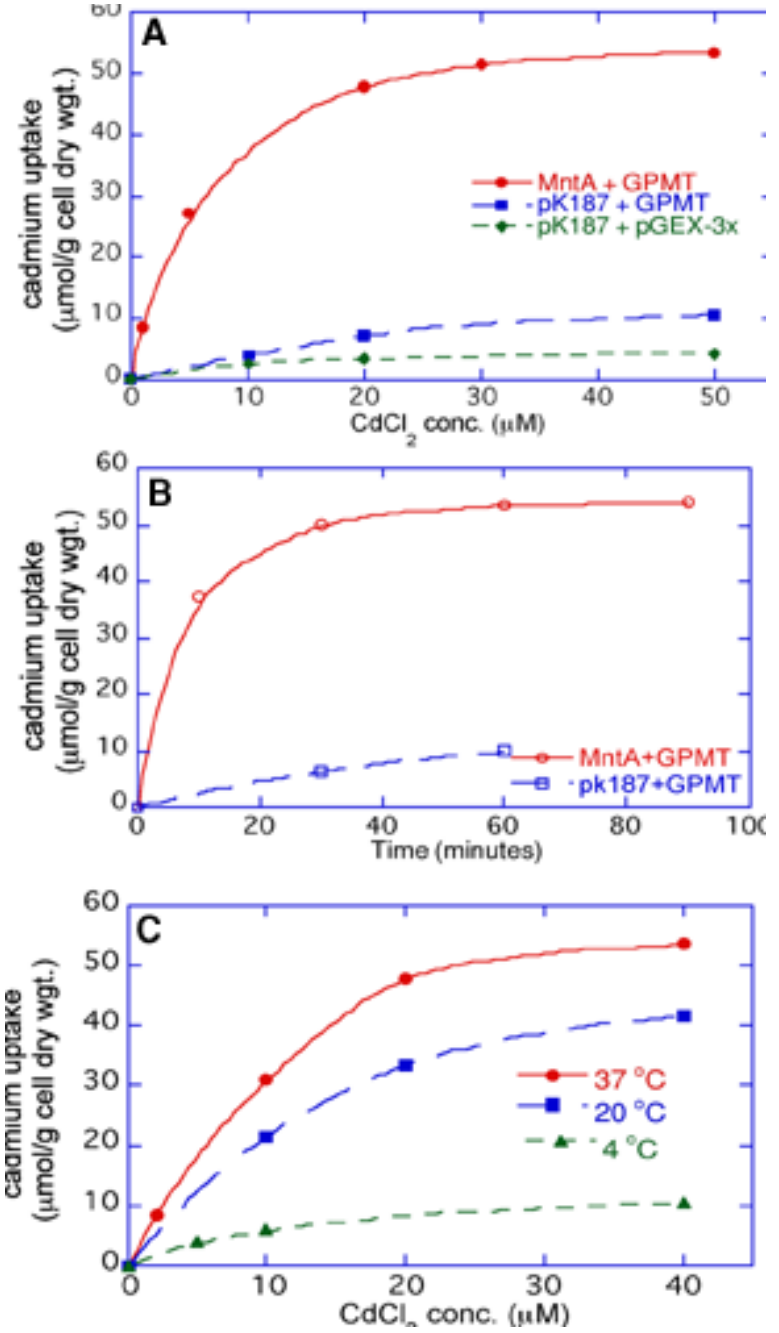

Fig. 2. Cellular accumulation of cadmium and copper at varying metal concentrations **(A,D)**, incubation times **(B,E)**, and incubation temperatures **(C,F)**. Induced cells containing both a P-type ATPase and an MT-fusion protein (MntA or CopA/GPMT), MT-fusion protein alone (pK187/GPMT), or neither (pK187/pGEX-3x) were incubated in 10 m*M* phosphate buffer for 30 min at 37°C in the indicated metal concentration unless otherwise noted. For time course studies (B,E), metal concentration was 20 µ*M* $CdCl_2$ (B) or $CuSO_4$ (E). Temperature studies (C,F) represent cells containing MntA/GPMT (C) or CopA/GPMT (F). Metal accumulation was determined by atomic adsorption spectroscopy *(continued on next page).*

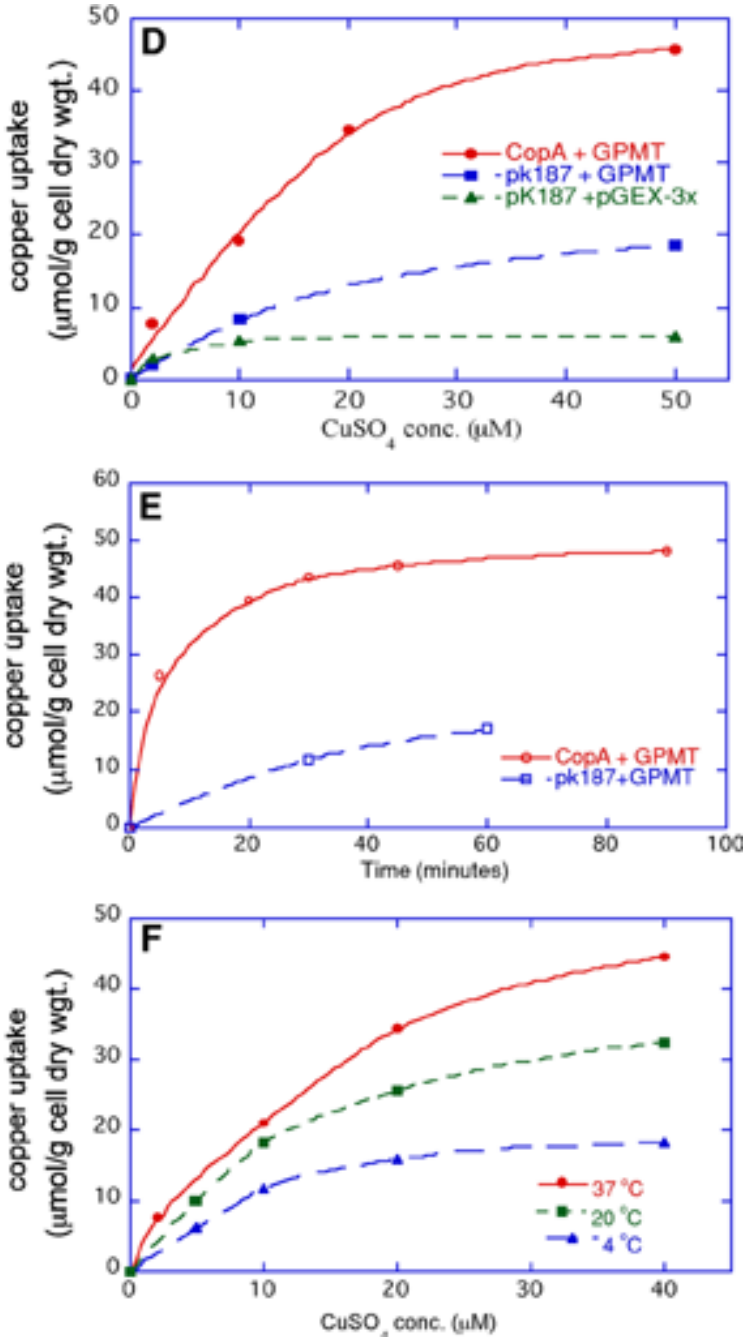

Fig. 2. *(continued)* Cellular accumulation of cadmium and copper at varying metal concentrations **(A,D)**, incubation times **(B,E)**, and incubation temperatures **(C,F)**. Induced cells containing both a P-type ATPase and an MT-fusion protein (MntA or CopA/GPMT), MT-fusion protein alone (pK187/GPMT), or neither (pK187/pGEX-3x) were incubated in 10 m*M* phosphate buffer for 30 min at 37°C in the indicated metal concentration unless otherwise noted. For time course studies (B,E), metal concentration was 20 µ*M* $CdCl_2$ (B) or $CuSO_4$ (E). Temperature studies (C,F) represent cells containing MntA/GPMT (C) or CopA/GPMT (F). Metal accumulation was determined by atomic adsorption spectroscopy.

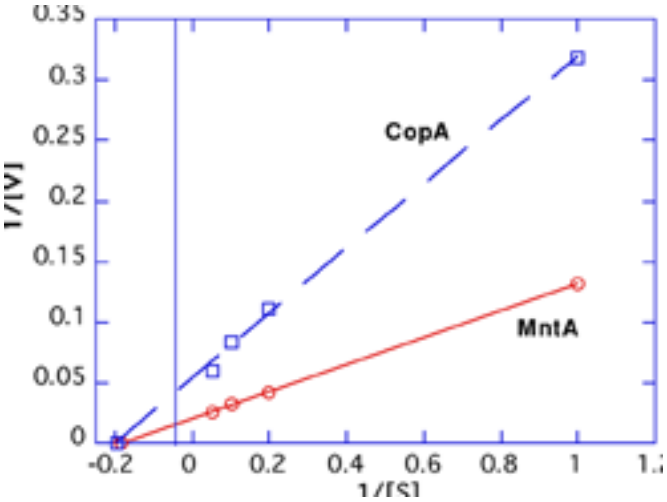

Fig. 3. Michaelis-Menten plot of CopA/GPMT and MntA/GPMT accumulation rate. Velocity rates vs substrate concentration were determined by plotting cellular accumulation of MntA/GPMT or CopA/GPMT minus the background accumulation obtained by pK187/GPMT (Fig. 2A, D).

The actual values for K_m and V_{max} were not determined, owing to the complexity of this system. Using this transporter/GST-MT system, cellular bioaccumulation can be measured, which indirectly relates to transport velocity. However, the rate of accumulation is dependent not only on translocation rate of the metal ions, but also rate of binding to metallothionein, amount of cellular ATP, extracellular metal concentration, and endogenous *E. coli* systems for metal influx or efflux.

Effects of Alkaline Metals

Alkaline metals such as Na^+ and Mg^+ are common components of many environments, and ionic strength can influence transporter activity. To determine the effect of ionic strength on copper and cadmium accumulation, cells were incubated in various concentrations of sodium chloride, magnesium chloride, and calcium chloride. As shown in Fig. 4, both sodium and magnesium inhibited the MntA transporter. Increasing sodium ion concentrations caused a steady decline in cadmium uptake, resulting in about 50% activity in the presence of 50 m*M* NaCl. Magnesium had a more pronounced effect; the addition of 1 m*M* $MgCl_2$ diminished cadmium bioaccumulation by >80%. However, concentrations above 1 m*M* did not cause any further significant decline in uptake. By contrast, sodium and magnesium had very little effect on the CopA transporter. Sodium had a very minor competitive effect on copper accumulation, and at 50 m*M* NaCl, total accumulation was reduced by about 20%. The addition of 1 m*M* $MgCl_2$ also had a relatively minor effect. Precipitation problems with $CaCl_2$ in buffer limited the concentration to a maximum of 1 m*M*, and from 0 to 1 mm

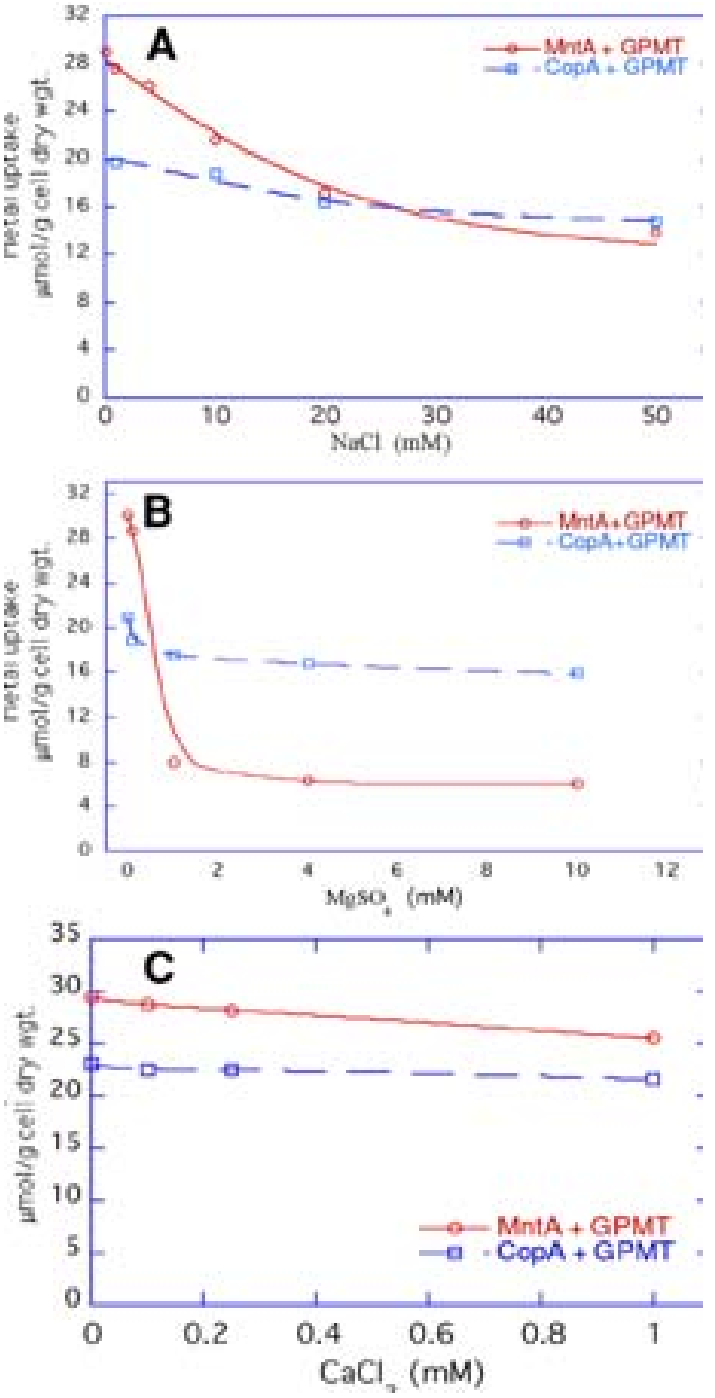

Fig. 4. Effect of sodium **(A)**, magnesium **(B)**, and calcium **(C)** on copper or cadmium accumulation. Induced cells containing ATPase and GST-MT plasmids were incubated in 10 m*M* phosphate buffer, pH 7.0, at 37°C for 30 min along with the indicated amounts of salt solutions and 10 µ*M* $CuSO_4$ (CopA) or $CdCl_2$ (MntA). Intracellular metal uptake was measured by atomic absorption spectroscopy.

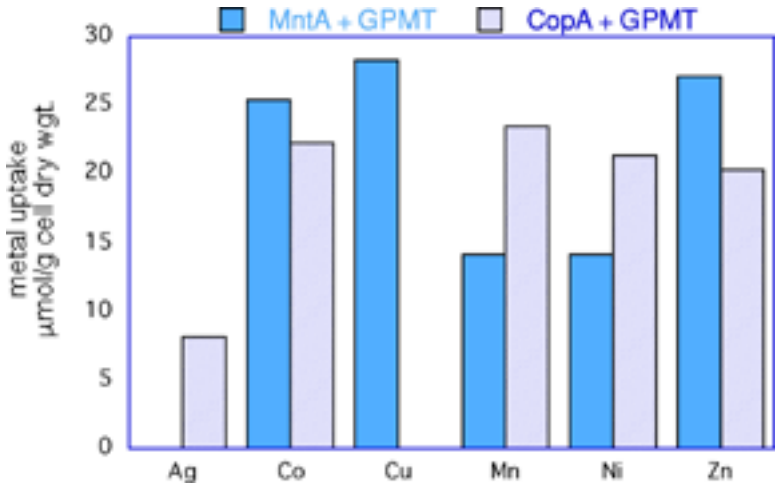

Fig. 5. Effect of competitive heavy metals on copper or cadmium accumulation. Induced cells containing ATPase and GST-MT plasmids were incubated in 10 m*M* phosphate buffer, pH 7.0, at 37°C for 30 min along with 10 µ*M* $CuSO_4$ (CopA) or $CdCl_2$ (MntA) and 50 m*M* $AgNO_3$ (Ag); 100 m*M* $CoCl_2$ (Co); 100 m*M* $CuSO_4$ (Cu); 100 m*M* $MnSO_4$ (Mn); 100 m*M* NiCl (Ni); or 100 m*M* $ZnSO_4$ (Zn). Intracellular metal uptake was measured by atomic absorption spectroscopy.

there was very little inhibition of uptake for either transport system, although the decrease was slightly larger with MntA.

Effects of Heavy Metals

To examine the specificity of the two transport systems, the cells were incubated in the presence of other heavy metals at 100 µ*M* concentrations with the exception of silver, which was shown to be toxic at that level. Figure 5 shows that both systems have a high degree of specificity. CopA was inhibited by silver, and MntA was inhibited by manganese, but these results would be expected since both metals have been shown to be substrates for the respective ATPases. In addition, even though manganese and silver were in excess, accumulation was only reduced about 50% for both MntA and CopA, confirming that these two systems have higher affinity for cadmium and copper, respectively. The MntA transporter was also slightly inhibited by the presence of nickel. It is possible that nickel could also be a substrate of the transporter.

Effects of Chelators and Complexing Agents

Various complexing agents are found in aquatic environments, such as silt, dissolved organic matter, or synthetic compounds. To test the effects of complexing agents on accumulation, cells were incubated in the presence of 1 m*M* sodium citrate, a weaker complexing agent, or EDTA, a stronger complexing agent *(18)*. As shown in Fig. 6, EDTA greatly reduced accumulation in both MntA and CopA cells. However, citrate, the weaker

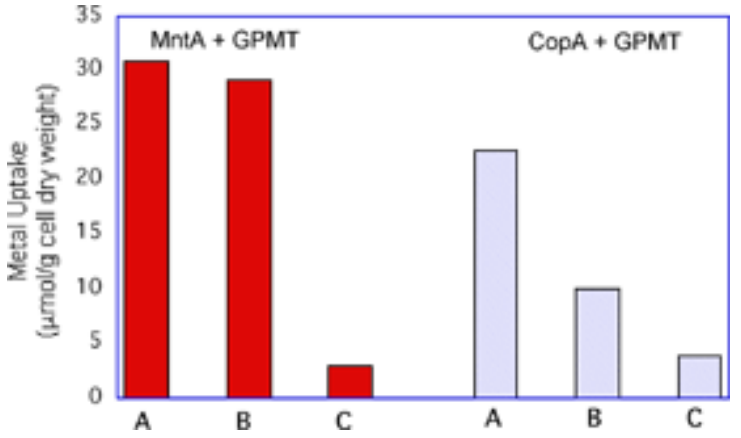

Fig. 6. Effect of metal chelators on copper or cadmium accumulation. Induced cells containing ATPase and GST-MT plasmids were incubated in 10 m*M* phosphate buffer, pH 7.0, at 37°C for 30 min along with 10 µ*M* $CuSO_4$ (CopA) or $CdCl_2$ (MntA) and no extra (A), 1.0 m*M* sodium citrate (B), or 1.0 m*M* EDTA (C). Intracellular metal uptake was measured by atomic absorption spectroscopy.

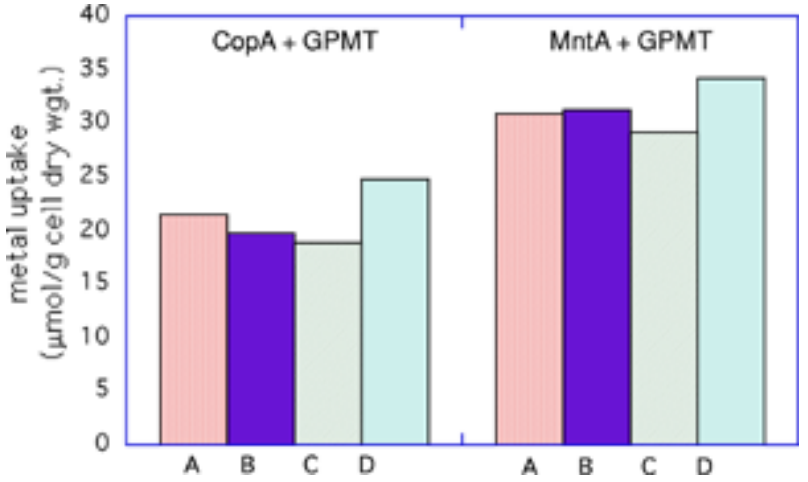

Fig. 7. Energy dependence of copper or cadmium accumulation. Induced cells containing ATPase and GST-MT plasmids were incubated in 10 m*M* phosphate buffer, pH 7.0, at 37°C for 30 min along with 10 µ*M* $CuSO_4$ (CopA) or $CdCl_2$ (MntA) and no extra (A), 1.0 m*M* KCN (B), 1.0 m*M* 2,4-DNP (C), or 0.4% glucose (D). Intracellular metal uptake was measured by atomic absorption spectroscopy.

chelator, reduced CopA activity by about 50% but did not affect accumulation in MntA.

Respiration Dependence of Transporters

Since both MntA and CopA are ATPases, and therefore energy dependent, uptake experiments were performed to determine the dependence on

Table 1
ATP Levels of CopA/GPMT Cells Under Different Incubation Conditions[a]

Incubation conditions	Cellular ATP (pmol/1 × 10^8 cells)
10 m*M* Buffer, 0 min	49.95 ± 1.30
Buffer + 10 μ*M* $CuSO_4$, 15 min	23.15 ± 0.45
Buffer + 10 μ*M* $CuSO_4$, 30 min	19.25 ± 0.70
Buffer + 1 m*M* 2,4-DNP, 15 min	24.65 ± 0.50
Buffer + 1 m*M* 2,4-DNP, 30 min	6.30 ± 1.60
Buffer + 10 μ*M* $CuSO_4$ and 1 m*M* DNP, 30 min	2.65 ± 0.55

[a]Induced cells containing the CopA and GPMT plasmids were incubated with the indicated solutions in 10 m*M* phosphate buffer, pH 7.0. At the appropriate time, 1 mL of cell solution was spun down, washed, and resuspended in ATP buffer. ATP levels were then measured by luciferin/luciferase assays as described in the text.

available oxygen or energy sources. Cells were incubated in the presence of either 1.0 m*M* KCN (a respiratory inhibitor) or 1.0 m*M* DNP (a respiratory uncoupler) to deplete energy, or with 0.4% glucose to provide additional energy. Figure 7 shows that the addition of glucose did stimulate activity for both CopA and MntA, resulting in a 15–20% increase in uptake. On the other hand, neither KCN nor DNP had any significant inhibitory effect on copper or cadmium accumulation. As depicted in Fig. 1B, cells showed a rapid initial uptake of metal, and most accumulation occurs in the first 10 min. The lack of DNP or KCN inhibition is likely owing to the high amount of accumulation that occurs before they affect cellular respiration. To test this, cells were preincubated with KCN or DNP for 15 min before addition of the metal substrate. In this case, a decrease in metal uptake was observed (data not shown), with DNP having a greater effect. Cellular ATP levels in these cells were examined as well, and they also confirmed that the effects of the respiratory inhibitors are not rapid (Table 1). When cells were exposed to metal solution, a majority of the ATP was used up in the first 15 min of accumulation, whereas DNP or KCN depleted ATP in the second 15 min.

pH Profile of MntA and CopA

The pH of an environment can play a large role in metal accumulation, because it can influence the speciation and mobility of a metal. At high pH, metals can form stable complexes with hydroxides, oxides, and other negatively charged groups, thus reducing bioavailability. At low pH, the presence of excess hydrogen atoms will cause cationic competition that can reduce uptake effectiveness. The effect of pH on MntA and CopA was examined across a broad range (pH 3.0–11.0) (Fig. 8). MntA displayed a broad range of maximum activity, from pH 4.0–11.0, with a significant drop-off at pH 3.0. CopA displayed a less-broad range of activity, with a maximum between pH 6.0–8.0. Low pH drop-offs are likely owing to the

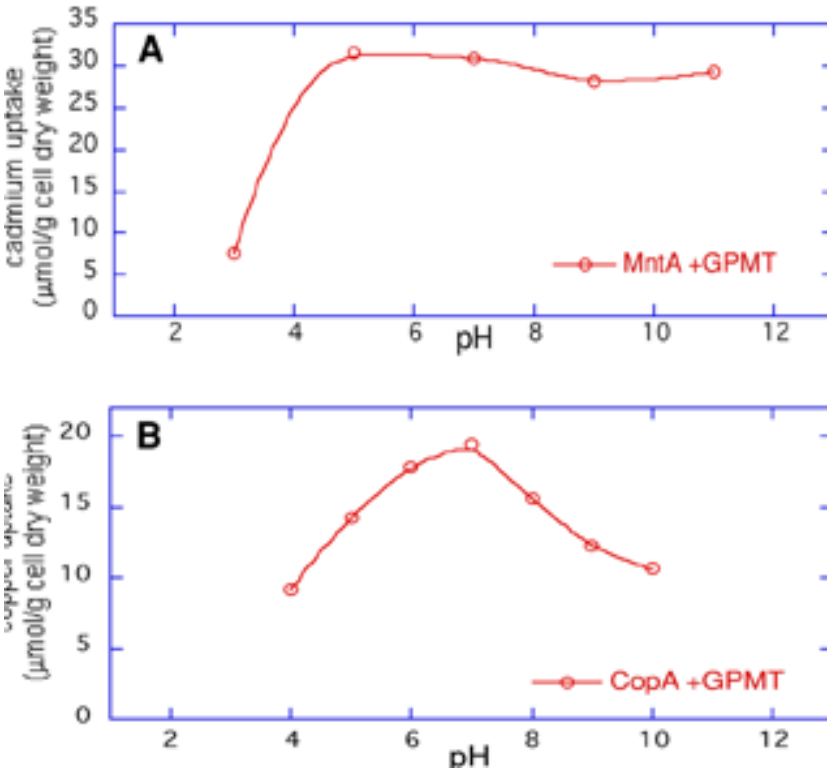

Fig. 8. pH profiles of MntA **(A)** and CopA **(B)** accumulation. Induced cells containing ATPase and GST-MT plasmids were incubated in 10 m*M* phosphate buffer adjusted to the indicated pH at 37°C for 30 min along with 10 µ*M* $CuSO_4$ (CopA) or $CdCl_2$ (MntA). Intracellular metal uptake was measured by atomic absorption spectroscopy.

interference of H^+ ions. Since the MntA-binding region involves carboxyl groups, which have a lower pK than the cysteines of CopA, it is not unexpected that CopA would be more sensitive to lower pH.

Conclusion

In terms of applicability to bioremediation, both engineered strains, CopA and MntA, show good promise. They are highly specific, show rapid accumulation, and can accumulate metal ions effectively in dilute solutions. In addition, all the requirements for accumulation are inside the cells; additional nutrients or energy is not required. However, both systems do have some shortcomings and are not as ideally suited as the genetically engineered Hg-accumulating strain that was constructed earlier *(19)*. The two particular areas of concern are the narrow pH range of the CopA ATPase, and the high magnesium sensitivity of MntA.

The differences in the two ATPases are interesting and provide some clues to the structure and function relationships of P-type ATPases. Despite the structural differences in these ATPases, it is believed that the mecha-

nism of binding and transport is likely the same, and therefore the maximum turnover might be similar. However, differences in the V_{max} were evident. Whereas these two membrane proteins have the same general function, the nutritional requirements for copper and manganese are not similar. Although typical bacterial heavy metal concentrations are in the micromolar range, the MntA transporter is utilized by *L. plantarum* to maintain intracellular manganese concentrations as high as 30 m*M*. Such intracellular concentrations are more typically associated with small essential ions such as Na or Mg. Structurally, MntA has significant homology to the non-heavy metal P-type ATPases that transport such small ions. It is possible that this structural configuration, with 10 transmembrane helices as opposed to 8, might increase the V_{max} for the MntA ATPase.

The structural similarities to sodium or magnesium pumps could also be responsible for MntA's sensitivity to these small ions. The binding site might be very similar, and the hydroxyl and carboxyl side chains involved in heavy metal binding are more favorable for interactions with hard Lewis acids than the cysteines of CopA and other heavy metal ATPases. However, the MntA protein also has significant homology to various H^+ ATPases, but increasing H^+ concentrations did not have any adverse effects on accumulation until the pH was below 4.0, so this may not be the underlying cause.

We have shown that designing bacterial systems that coexpress a membrane transporter and metallothionein provide a simple, although indirect, method to study heavy metal transport across membranes and compare accumulation in different transporters under similar conditions. Four different systems have been set up, and experimental data can be combined with sequence and structural data to gain insights into the underlying biology of these transporters. As more ATPases are studied, we can understand what properties are specific to the protein and which ones may be related to the family, or subfamily, as a whole. Understanding the molecular mechanisms involved should make it easier to use genetic engineering to improve on the intrinsic transport activity of these systems, as well as possibly to design novel ones in the future.

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