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INDUCTION OF THE PUTATIVE COPPER ATPases, CopA AND CopB, OF *Enterococcus hirae* BY Ag^+ AND Cu^{2+}, AND Ag^+ EXTRUSION BY CopB

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The two P-type ATPases CopA and CopB are effecting regulation of cellular copper activity in *Enterococcus hirae*. With antibodies against these ATPases, we showed on Western blots the simultaneous induction of CopA and CopB by copper or silver ions. Copper contents of wild type and mutant cells lacking either CopA, CopB or both enzymes were measured by atomic absorption. Strains disrupted in *cop*B showed clearly enhanced copper contents. Mutants lacking CopB also lost the ability of energy dependent efflux of silver ions. Our results demonstrate that CopA and CopB are under the same genetic control and support the proposal that CopB is a copper and silver exporting ATPase.

The essential trace element copper is a cofactor of many enzymes involved in redox reactions, such as cytochrome c oxidase, ascorbate oxidase, or superoxide dismutase. But the oxidative potential of copper can also cause extensive cellular damage through formation of free radicals and direct oxidation of proteins, lipids, nucleic acids and polysaccharides. The control of cytoplasmic copper is therefore crucially important for cell viability.

In the Gram-positive bacterium *Enterococcus hirae*, two P-type ATPases, CopA and CopB, appear to be involved in the regulation of intracellular copper activity (1). Both enzymes exhibit extensive sequence identity to the three putative copper pumps encoded by the human Menkes gene (2), its murine homologue (3) and the human Wilson gene (4). All five proteins are members of the P-type ATPase family, suggesting that copper-transporting ATPases represent a subclass of these enzymes (5). CopA, the Menkes and the Wilson ATPase contain the same conserved heavy metal binding motifs also found in the cadmium efflux ATPase CadA of *Staphylococcus aureus* (6), and in proteins involved in mercury resistance (7,8). In contrast, CopB of *E. hirae* contains a copper binding motif that is also present in otherwise unrelated proteins. Namely in the periplasmic copper binding proteins CopA and CopC of *Pseudomonas syringae* (9,10) and in two proteins, CutE and PcoD, that are involved in copper metabolism in *Escherichia coli* (11,12). A very similar motif also occurs in the recently discovered copper uptake protein CTR1 of the yeast *Saccharomyces cerevisiae* (13).

Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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Although the presence of copper binding motifs in these ATPases suggests that they are copper pumps, transport across the membrane has not yet been directly demonstrated for any of these ATPases. We here present evidence that CopA and CopB of *E. hirae* are induced by Cu^{2+} and Ag^{+} and that CopB can export silver ions from the cytoplasm.

MATERIALS AND METHODS

Materials—Enterococcus hirae (ATCC9790, formerly called *Streptococcus faecalis* or *faecium*) was obtained from the American Type Culture Collection. Antibodies against CopB and the null-mutants Δ*cop*A, Δ*cop*AΔ*cop*B and Δ*cop*B of *E. hirae* have been described previously (1). Growth media additives were purchased from BBL, [^{110}Ag]silver nitrate from Amersham, LUMA-Gel scintillation cocktail from LUMAC, the Netherlands. All other molecular biology reagents and materials were obtained from Boehringer-Mannheim. Other chemicals were bought from Sigma and were of the highest grade available.

Protein expression—Cells were grown to 0.7 OD units (546 nm) in 1 ml of 1% $Na_2HPO_4 \cdot 2H_2O$, 1% trypticase peptone, 0.5% yeast extract, and 1% glucose. Following induction with $CuSO_4$ or $AgNO_3$ for 1 h under the same conditions, cell extracts were prepared by centrifuging the cultures and adding to the cell pellet 50μl of 10 mg/ml lysozyme, 1 mM EDTA, 10 mM Tris-Cl, pH 8. After incubation for 10 min at room temperature, 10 μl of 1 mg/ml DNase I in 100 mM $MgCl_2$ were added, and incubation continued for 5 min. Samples of these extracts containing the same amount of protein were separated on sodium dodecyl sulfate gels (14), which were subjected to Western blotting as described (15). CopA could not be detected if defatted milk was used in place of bovine serum albumin for quenching the blots.

Antibodies against CopA—The DNA sequence coding for the 457 C-terminal amino acids of CopA was excised from the original clone pZ3 (16) with *Spe*I and *Eco*RI, made blunt ended with Klenow polymerase and cloned into the Klenow filled *Bam*HI site of pEX3 to generate a β-galactosidase-CopA fusion protein. Isolation of the fusion protein and generation of rabbit polyclonal antibodies have been described (17).

Determination of copper content—Cells were grown to 0.8 OD units (546 nm) and $CuSO_4$ added to a final concentration of 1 mM. 1 h later cells were harvested by centrifugation, washed three times with 10 mM Tris-Cl pH 8, 2 mM $MgSO_4$. The pellets, corresponding to 7 mg of dry cell weight, were suspended in 1 ml of 10% nitric acid, 10% perchloric acid and boiled for 30 min. Copper content was determined with an atomic absorption spectrophotometer Perkin Elmer 2380 at 324.8 nm.

Silver extrusion—Cells were grown to 1 OD unit (546 nm), washed with 0.2 and 0.1 volume of ice cold 200 mM K^+-HEPES, 5 mM $MgSO_4$, pH 7.4 and suspended in 0.01 volume of the same buffer. Silver loading of the cells was conducted as follows: 50 μl of washed cells were added to 450 μl 10 mM K^+-HEPES, 2 mM $MgSO_4$, pH 7.4, 15 μM $AgNO_3$ (^{110}Ag: 82.4 nCi/μg silver) and incubated for 40 min at 37°C. To start extrusion, 10 μl 50% glucose was added. Aliquots of 100 μl cells, corresponding to a dry cell weight of 290 μg were filtered through 0.45 μm nitrocellulose filters and immediately washed twice with 1 ml ice cold 10 mM K^+-HEPES, 2 mM $MgSO_4$, 1 mM $AgNO_3$. The nitrocellulose filters had been preincubated with 10 mM K^+-HEPES, 2 mM $MgSO_4$, 100 μM $AgNO_3$ for at least 1 h. For β-counting, the filters were dissolved in 5 ml LUMA-Gel scintillation cocktail.

RESULTS AND DISCUSSION

The *cop* operon of the Gram positive bacterium *Enterococcus hirae* encodes the two P-type ATPases CopA and CopB, that are involved in the regulation of intracellular copper activity. Based on growth studies with wild type and mutant cells, it was previously suggested that CopA serves in the uptake and CopB in the extrusion of copper (1).

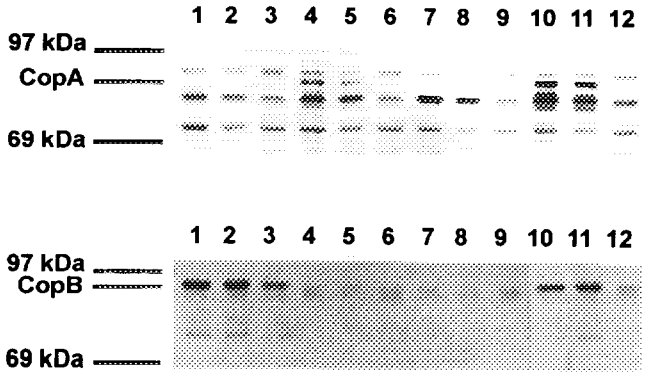

Figure 1. **Induction of CopA and CopB by copper and silver ions.** *E. hirae* cells were induced in the logarithmic phase with the respective ion for 1 h and cell lysates tested for the expression of CopA (upper panel) or CopB (lower panel) on Western blots with antisera against CopA or CopB, respectively. Details are described under "Materials and Methods". The migration of marker proteins of 97 kDa and 69 kDa and of CopA and CopB are indicated. Lanes 1-3, mutant Δ*cop*A; lanes 4-6, mutant Δ*cop*B; lanes 7-9, mutant Δ*cop*AΔ*cop*B; lanes 10-12, wild type. Each strain is shown, in this order, induced with 20 μM $AgNO_3$, induced with 2 mM $CuSO_4$ and uninduced, respectively.

With antibodies raised against CopA and CopB respectively, both ATPases could be detected on Western blots (Fig. 1). The electrophoretic mobilities of CopA and CopB corresponded to M_r values of 87000 and 93000. In contrast, the calculated M_r values for CopA (727 amino acids) and CopB (745 amino acids) are 78387 and 81522 and both ATPases thus exhibit a decreased mobility on sodium dodecyl sulfate gels. Both enzymes were induced by high concentrations of copper or silver ions with maximal induction reached at 2 mM $CuSO_4$ or at 20 μM $AgNO_3$. Maximal expression of both proteins could also be observed at very low levels of free copper ions in the media, generated by adding 200 μM of the strong chelators *o*-phenanthroline or 8-hydroxyquinoline (not shown). The simultaneous induction of CopA and CopB is in line with the arrangement of their genes on the same operon, and therefore underlying the same control. However, the promoter region of the *cop* operon has not yet been cloned and characterized.

The copper content of wild type and mutant cells, grown for one hour in medium containing 1 mM $CuSO_4$, was determined by atomic absorption measurements (Fig. 2). Only approximately 0.1 % of the copper initially present in the medium could be detected in the cells. The mutant Δ*cop*B that does not express the CopB ATPase and mutant Δ*cop*AΔ*cop*B that expresses neither the CopA nor the CopB ATPase accumulated copper to a three fold or higher extent than strains containing a functionally expressed CopB. While wild type and mutant Δ*cop*A grow in media supplemented with up to 8 mM $CuSO_4$, the mutants defective in CopB have a copper-sensitive phenotype with reduced growth even at 1 mM $CuSO_4$ (not shown). These results support the concept that CopB serves in the extrusion of copper.

Ag^+ has been shown to replace Cu^+ in some processes. Ag^+ was used in studies to determine the stoichiometry and coordination properties of metallothionein (18) or in the characterization of the yeast ACE1 transcription factor (19). Since silver ions can not undergo

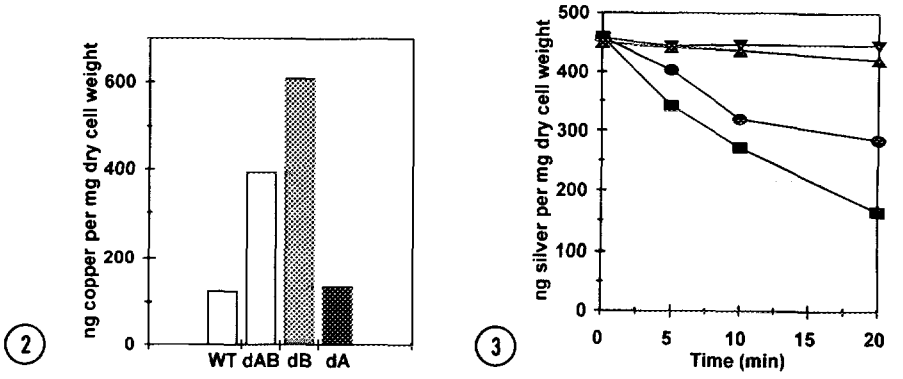

Figure 2 **Copper content of wild type and mutant cells.** Accumulated copper in cells grown for 1 h at 1 mM $CuSO_4$ was measured by atomic absorption. Details of the procedure are given under "Materials and Methods". Open bar (WT), wild type; dotted bar (dAB), Δ*cop*AΔ*cop*B mutant; cross hatched bar (dB), Δ*cop*B mutant; solid bar (dA), Δ*cop*A mutant.

Figure 3 **Energy dependent silver extrusion from silver loaded wild type and mutant cells.** Cells were loaded with radioactive [^{110}Ag]silver. Glucose was added at time zero to start energy dependent extrusion. Aliquots of the cell suspension corresponding to the same dry cell weight were filtered, washed and the amount of silver determined as described in "Materials and Methods". ●, wild type; ■, Δ*cop*A mutant, ▲, Δ*cop*B mutant; ▼, Δ*cop*AΔ*cop*B mutant.

the reversible redox reactions of copper, it is a very toxic ion by inhibiting copper containing redox enzymes.

E. hirae is a facultative anaerobic bacterium, which gains its energy mainly by fermentation of glucose and does not possess significant energy stores (20). Cells devoid of an energy source could thus be loaded with radioactive Ag^+ at a total concentration of 15 μM, corresponding to 570 ng Ag^+ per mg dry cell weight. During loading, all strains investigated bound about 450 ng Ag^+ per mg dry cell weight, nearly 80% of the total Ag^+ (Fig. 3). Wild type cells actively extruded Ag^+ when glucose was supplied. About 50% of the loaded silver was exported 20 minutes after energizing the cells by adding glucose. Mutants lacking CopA were still able to extrude Ag^+, even at a slightly increased rate compared to wild type. In contrast, cells deficient in CopB had lost the ability of energy dependent Ag^+-extrusion. Previous experiments have shown that disruption of *cop*A renders the cells more resistant to Ag^+, suggesting a role of CopA in import (1). Hence the lower rate of Ag^+-extrusion observed in wild type cells may be due to a toxic effect of the ion. If the loading procedure was conducted at silver concentrations higher than 15 μM, decreased transport rates were observed for both, wild type and Δ*cop*A mutant cells, in line with the proposed toxic effect of silver (not shown).

In this report we show that the expression of CopA and CopB underlay the same control. Both are inducible by high ambient copper or silver concentrations. Mutants lacking CopB show significantly higher copper contents. They are also no longer able to extrude Ag^+. These results strongly support the proposal that CopB serves in the extrusion of heavy metal ions from the cytoplasm. That CopB is responsible for the extrusion of monovalent silver ions

would suggest that it functions as a pump for monovalent rather than divalent heavy metal ions.

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