

## Copper homeostasis in *Enterococcus hirae*: pumps, repressor, chaperone

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*Enterococcus hirae* is a convenient model system for the study of copper homeostasis. Its *cop* operon encodes two copper ATPases, CopA and CopB, that serve in the import and the export of copper(I), respectively. The copper chaperone, CopZ, effects the intracellular routing of copper and the copper responsive repressor, CopY, regulates the expression of the four genes. All components of the system have been purified and are under structural and functional investigation.

Since the first report of ATP-driven copper transport in the Gram-positive *Enterococcus hirae* in 1992 [1], our understanding of cellular copper metabolism has virtually exploded. Today, we have molecular information on key steps and components of cellular copper homeostasis, stemming from the study of bacteria, yeast and mammalian cells. In the unraveling of cellular copper circulation, *E. hirae* has proven to be an excellent model system [2].

In *E. hirae*, the *cop* operon encodes four key components of copper metabolism, namely two copper pumps, a repressor, and a copper chaperone. The two copper ATPases, CopA and CopB, accomplish transmembranous copper transport, with CopA serving in the uptake of copper under limiting conditions, and CopB in copper secretion when it reaches toxic levels [3]. CopA and CopB share 35 to 40% sequence identity with the human copper ATPases which are associated with two inherited disorders of copper metabolism, Menkes and Wilson disease, respectively, but also with cadmium ATPases of bacterial resistance systems.

Based on the protein sequences, CopA, CopB, cadmium ATPases, and the human copper ATPases are P-type ATPases, classically represented by Ca- and Na,K-ATPases. P-type ATPases share the conserved sequence motif DKTGT. The aspartic acid residue of this motif forms a high-energy acylphosphate intermediate in the course of the reaction cycle - hence the name P-type ATPases. As more copper ATPases were discovered, it became apparent that they, together with the cadmium ATPases, had a secondary structure and a predicted membrane topology quite distinct from the non-heavy metal ATPases. Phylogenetic analysis revealed that the ion-motive ATPases branched into heavy metal and non-heavy metal ATPases early in evolution, probably before the division into prokaryotes and eukaryotes. To date, over 30 heavy metal ATPases have been cloned and ion specificities include Cu<sup>+</sup>,

Ag<sup>+</sup>, Zn<sup>2+</sup>, Cd<sup>2+</sup>, and Pb<sup>2+</sup>. Due to their different structures, the heavy metal ATPases have been assigned to a subclass of the P-type ATPases, called CPx-type or P1-type ATPases [4,5].

The evidence for copper uptake through the action of the *E. hirae* CopA ATPase is so far indirect: first, knock-out mutants in CopA are unable to grow in copper depleted media and, secondly, such mutants can grow in 5 μM AgNO<sub>3</sub>, which completely inhibits the growth of wild-type cells [3]. This suggests that CopA serves as a pathway for the entry of copper(I), but also silver(I), into the cell. CopA ATPase endowed with an N-terminal, cleavable histidine-tag was expressed in *E. coli* and purified to homogeneity by affinity chromatography on a nickel resin. Isolated CopA, when reconstituted into proteoliposomes, formed an acylphosphate intermediate and therefore belongs to the supergroup of P-type ATPases. The purified, reconstituted CopA ATPase serves as an ideal model system for the study of ATP-driven copper transport.

The function of CopB of *E. hirae* in copper export is supported by three lines of evidence. First, CopB knock-out mutants are sensitive to micromolar concentrations of copper, while wild-type *E. hirae* tolerates up to 8 mM copper. Secondly, when cells are loaded with <sup>110m</sup>Ag<sup>+</sup>, ΔCopB mutants do not extrude the silver while wild-type cells do. Third, native inside-out membrane vesicles of *E. hirae* exhibit ATP-driven accumulation of copper(I) or silver(I) [6]. Thus, CopB appears to serve in the extrusion of copper from cells if copper is excessive. Silver(I), which has an ionic radius similar to that of copper(I), is a substrate for both, the CopA and the CopB ATPase.

CopB was purified from an *E. hirae* mutant with up-regulated expression of the *cop* operon. Since the N-terminus of CopB is very rich in histidine residues, it could be affinity purified on a nickel affinity resin without the need of adding a histidine tag. Purified CopB, reconstituted into proteoliposomes, formed an acylphosphate intermediate, was inhibited by vanadate, and showed a limited copper dependence [7].

The CopB experimental system proved valuable for the analysis of the functional role of amino acid residues. A CPH or CPC motif is found in the sixth transmembranous helix of all CPx-type ATPases (hence the name) and is believed to serve in the translocation of ions across the membrane. The cysteine of the CPH motif of CopB was mutated to serine (C396S). This mutation abolished copper transport function *in vivo*, as assessed by the complementation of a CopB knock-out strain. However, C390S-CopB was still able to form an acylphosphate intermediate from ATP. The partial function of this mutant enzyme indeed suggests a direct role of the CPH motif in transport, but not in ATP hydrolysis.

CopY is a copper responsive repressor that regulates the expression of the *cop* operon. In normal media that contain approximately 10 μM copper, expression of the *cop* genes is minimal. Higher as well as lower copper levels induce the operon. We could show that this regulation was effected by the binding of CopY to two binding sites upstream of the coding region [8]. Mutation of both CopY binding sites to weaken CopY binding, lead to hyperinduction by excess copper, but did not affect induction by a lack of copper. This suggests that two different mechanisms are responsible for the induction of the *cop* operon by high and low copper [9].

Since copper ions can undergo a Fenton type reaction with the generation of radicals, copper ions are probably never free in the cytoplasm. This requires specialized proteins to

route copper intracellularly [10]. In *E. hirae* CopZ appears to fulfill this role. With an *in vitro* band-shift assay, we could show that copper(I)-CopZ can donate copper to the CopY repressor, thereby releasing it from the DNA. This copper transfer appeared to be specific, as a similar copper binding protein was unable to donate copper to CopY [11].

The structure of CopZ was elucidated by NMR spectroscopy. It exhibits a  $\beta\alpha\beta\beta\alpha\beta$ -fold, with the four  $\beta$ -strands forming an antiparallel  $\beta$ -sheet and the two  $\alpha$ -helices lying on top of it. The copper binding motif CxxC lies between the first  $\beta$ -strand and the beginning of the first  $\alpha$ -helix [12]. This structure closely resembles those of the bacterial mercury chaperone MerP [13], the yeast copper chaperone Atx1 [14], and the fourth copper binding motifs of the human Menkes copper ATPase [15]. Upon the binding of copper, the NMR signals from the vicinity of the copper binding region were lost, probably due to dimerization of Cu(I)-CopZ. No major structural changes could be observed in the remainder of the protein.

Study of the *E. hirae* *cop* operon has so far considerably contributed to our understanding of copper homeostasis. Using purified components of this system promises to provide further insight into nature's dealing with copper.

## References

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