

# Effects of Promoter Mutations on the *in Vivo* Regulation of the *cop* Operon of *Enterococcus hirae* by Copper(I) and Copper(II)

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**The *cop* operon of *Enterococcus hirae* encodes a repressor, CopY, a copper chaperone, CopZ, and two copper ATPases, CopA and CopB. Regulation of the *cop* operon is bi-phasic, with copper addition as well as copper chelation leading to induction. Using a plasmid-borne system with a reporter gene, induction of wild-type and mutant *cop* promoters by high and low copper conditions was investigated. Only mutations that impaired the interaction of CopY with both DNA binding sites had a marked effect on regulation, leading to hyperinduction by copper(I) or copper(II). Chelation of copper(II), but not copper(I), also induced the operon, but induction by copper chelation was not significantly affected by the mutations. *E. hirae* mutants with reduced extracellular copper reductase activity exhibited the same induction kinetics as wild-type cells. These results show that copper addition and copper chelation induce the *cop* operon by different routes.** © 1999 Academic Press

Copper is an essential element through its functions as a cofactor in many redox enzymes. But copper is also very toxic to cells by damaging biomolecules through radical formation. Intracellular copper concentrations therefore need to be regulated within narrow limits (1). In the Gram-positive bacterium *Enterococcus hirae*, copper homeostasis is accomplished by the *cop* operon, which consists of the four genes *copY*, *copZ*, *copA*, and *copB* (2). *CopY* encodes a copper responsive repressor and *copZ* a copper chaperone that routes copper intracellularly (3, 4). The two large genes *copA* and *copB* encode CPx-type copper ATPases (5) and earlier data suggested that CopA serves in copper uptake under conditions of copper limitation, and CopB in its extrusion when intracellular copper reaches toxic levels (6,

7). Expression of the *cop* operon was shown to be regulated by ambient copper in a bi-phasic fashion: expression was minimal in standard growth media; addition as well as complexation of copper led to the induction of the operon (6). There was only one predominant mRNA species apparent under different induction conditions, arguing against the existence of internal promoter sites of significant strength (D. Stausak, unpublished).

Expression is apparently controlled by the CopY repressor. By DNaseI footprinting, it had been shown that CopY protected two regions of 27 and 28 bp featuring an inverted repeat and flanking the translational start site of the *cop* operon. Two independent CopY binding sites were corroborated by DNA band shift assays, where it was apparent that the CopY-DNA interaction occurred in two discrete steps by sequential binding of CopY to the two sites. The half-association concentrations of CopY for these sites *in vitro* were 5 nM for the operon-distal and 2 nM for the proximal site (3). CopY requires one zinc(II) ion for the binding to DNA (4). Copper(I) ions delivered to CopY with the copper chaperone CopZ *in vitro* cause the exchange of the bound zinc by two copper(I) and concomitant release of CopY from the promoter (4).

We here analyzed the induction of the *cop* operon *in vivo*, using plasmid-borne wild-type and mutant *cop* promoters and truncated CopB as a reporter gene. Mutation of both, but not one, CopY binding site on the promoter led to hyperinduction. Induction by excess copper or lack of copper was affected differently, suggesting different mechanisms of induction. An extracellular reductase that converts copper(II) to copper(I) was identified and its influence on induction investigated.

## MATERIALS AND METHODS

**Materials.** *Enterococcus hirae* (ATCC9790, formerly called *Streptococcus faecalis* or *faecium*) was obtained from the American Type Culture Collection. *E. coli* strains XLmutS and XL1-Blue were obtained from Stratagene Inc. Anti-CopB antibody and the  $\Delta copAB$

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strain B1 have been described previously (8). Fluorescein-isothiocyanate-conjugated swine anti-rabbit IgG was obtained from DAKO (Diagnostics AG, Switzerland). Bicinchoninic acid (BCA) was bought from Pierce. All other molecular biology reagents and materials were obtained Roche, Sigma and Merck and were of the highest grade available.

**Plasmid construction.** The  $P_{WT}$  reporter plasmid pOB2 was generated as follows: the Klenow-filled *EcoRI-HindIII* fragment of the *cop* promoter region from pOA1 (9) was cloned into the *E. coli/E. hirae* shuttle vector pC3 (10), also cut with *EcoRI* and *HindIII* and Klenow-filled. The resultant plasmid pOC1 was cut with *BamHI* and *SaII* and ligated with the 2844 bp *BamHI/SaII* fragment of the *copB* gene to complete it in the resultant plasmid pOB1. From this plasmid, pOB2 was obtained by deletion of the 770 bp *PstI-PstI* fragment, which truncated CopB by 80 amino acids at the C-terminus (truncated CopB, CopB665). The  $P_A$  reporter plasmid was generated similarly from pOM214 (3) that carried the A-61T mutation. To obtain the  $P_{AB}$  (A-61T/A-30T) reporter plasmid pOB361, pOM214 was mutated with the Chameleon mutagenesis kit (Stratagene) using the mutagenic primers 5'-GTTAAGTTTTCAAATGTAATCG-3' and the commercial switch-toggle *MluI-ScaI* primer. From the resultant plasmid pOM30, the 1710 bp *SacI/BamHI* fragment was subcloned into pOB2 cut with the same enzymes. Standard molecular biology methods were conducted by published procedures (11).

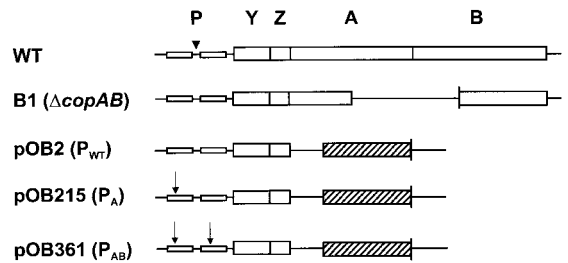
**Expression assays.** Cells were grown semi-anaerobically (capped, but not de-oxygenated tubes) to an  $OD_{546}$  of 0.3–0.5 in N-media (1%  $Na_2HPO_4 \cdot 2H_2O$ , 1% trypticase peptone, 0.5% yeast extract, 1% glucose). Following induction with the respective agents for 1h, cell extracts were prepared by centrifuging the cultures and adding to the cell pellets 50  $\mu$ l of 10 mg/ml lysozyme, 1 mM EDTA, 10 mM Tris-Cl, pH 8. After 10 min incubation at room temperature, the cells were taken through a freeze-thaw cycle, 10  $\mu$ l of 1 mg/ml DNaseI in 100 mM  $MgCl_2$  were added, and incubation continued for 5 min at room temperature. Amounts of these extracts corresponding to the same number of cells were separated on sodium dodecyl sulfate gels (12), followed by Western blotting as described (13), using phosphate-buffered saline, 10% horse serum, 0.1% Tween 20, for quenching. Anti-CopB antibodies (8) were used for detection of CopB and CopB665, and fluorescein-isothiocyanate-conjugated swine anti-rabbit IgG was used as second antibody. The fluorescence was measured quantitatively with a Phosphor Imager (Molecular Dynamics).

**Reductase assays.** Copper reductase activity in whole cells was determined spectrophotometrically at 37°C as described (14), but using the following buffer: 200 mM N-morpholinoethane sulfonic acid, 20 mM Na-citrate, 5 mM  $MgCl_2$ , 500  $\mu$ M  $CuSO_4$ , 500  $\mu$ M BCA, pH 6. Reactions were started by the addition of 1% glucose. An extinction coefficient of  $4.6 \times 10^6 M^{-1}cm^{-1}$  at 355 nm was used for the copper(I)-BCA complex (Cu(I)BCA). Iron reductase was measured similarly, using 500  $\mu$ M bathophenanthroline and 500  $\mu$ M  $FeCl_3$  and using an extinction coefficient for Fe(II)bathophenanthroline of  $2.2 \times 10^4 M^{-1}cm^{-1}$  at 520 nm (15).

**Reductase mutant selection.** *E. hirae* wild-type log cells were washed with 0.1 M NaP<sub>i</sub>, pH 7, mutagenized for 90 min with 1.6% ethyl methanesulfonate in the same buffer, followed by washing and plating on N-media (9). Colonies were transferred to 0.45  $\mu$ m nitrocellulose membranes and, colonies up, incubated on filter paper soaked with R-buffer, which was optimized for the plate assay (50 mM Na-citrate, 1 mM BCA, 1 mM  $CuSO_4$ , pH 6.5) containing 5% glucose. Within 40 min, colonies with reductase activity turned intensely pink, while reductase deficient mutants stained only faintly.

## RESULTS

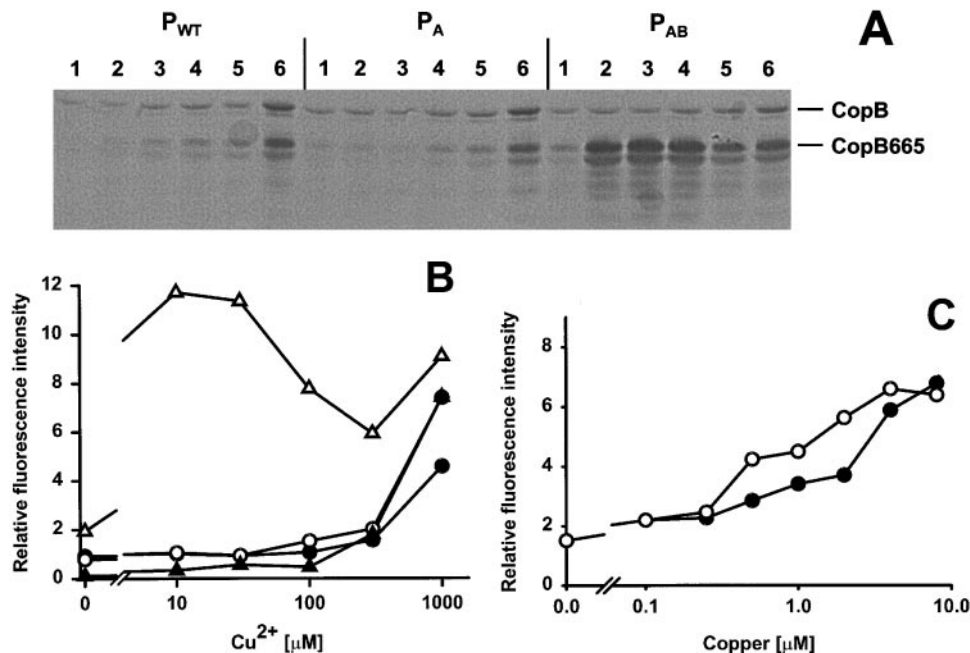
**Reporter gene assay for the analysis of the in vivo regulation of the cop operon.** To study the regulation of the *cop* operon by copper and the effects of promoter



**FIG. 1.** Schematic representation of the strains and plasmids used in this study. WT, genomic *cop* operon in wild-type *E. hirae*; B1, genomic *cop* operon in  $\Delta copAB$  strain; pOB2, pOB215, pOB361, plasmid-borne *cop* operons with CopB665 reporter gene and wild-type or mutant promoters. The small open boxes represent the two CopY repressor binding sites of the *cop* promoter (P). Transcription is initiated at the arrowhead. The *copY*, *Z*, *A*, and *B* wild-type genes are indicated by open boxes and the truncated *copB665* reporter gene by gray boxes. The erythromycin gene of the cassette used to generate the  $\Delta copAB$  strain B1 is depicted as a solid bar. Arrows point to the positions of promoter mutations used.  $P_{WT}$  designates the plasmid-borne wild-type promoter,  $P_A$  the plasmid-borne A-61T single mutant promoter, and  $P_{AB}$  the A-30T/A-61T double mutant promoter.

mutations, we constructed a plasmid-borne expression system with the following components: the *cop* promoter containing the repressor binding sites, the *copY* repressor gene, the *copZ* chaperone gene, and a reporter gene encoding a truncated form of CopB, CopB665 (Fig. 1). Attempts to use the common reporter genes chloramphenicol acetyltransferase or  $\beta$ -galactosidase in *E. hirae* had failed for unknown reasons. CopB665 lacked the C-terminal 80 amino acids and thus the two C-terminal transmembranous helices. In wild-type *E. hirae* cells, CopB functions in copper extrusion and thereby confers copper resistance, allowing the cells to grow in up to 8 mM  $CuSO_4$ ; cells deleted in CopB are growth-inhibited by more than 10  $\mu$ M  $CuSO_4$  (8). When CopB665 was expressed in the  $\Delta copAB$  deletion strain B1, the cells remained sensitive to above 10  $\mu$ M added copper, confirming that the truncated CopB665 ATPase could not function in copper extrusion.

**Induction of wild-type and mutant cop promoters by copper.** In Fig. 2, expression levels of CopB from the genomic wild-type promoter were compared to expression levels of the CopB665 reporter protein from plasmid-borne wild-type and mutant promoters in the same cell. Both, the genomic promoter and the plasmid-borne wild-type promoter ( $P_{WT}$ ) exhibited closely similar induction kinetics by copper. The one hour induction time in this and the following experiment had been chosen based on previous experiments that had shown that steady state levels of induction were reached one hour after induction, for both, CopB and CopB665 (16). Expression of genomic CopB was essentially the same in plasmid bearing and plasmid-free strains, indicating the absence of significant gene



**FIG. 2.** Copper induction of genomic CopB, and of CopB665 reporter protein from plasmid-borne wild-type and mutant promoters. (A) Western blot of wild-type *E. hirae* expressing CopB from the genomic wild-type promoter and CopB665 from a plasmid-borne wild-type promoter ( $P_{WT}$ ), the A-61T mutant plasmid promoter ( $P_A$ ), and the A-61T/A-30T double mutant plasmid promoter ( $P_{AB}$ ). Cells were induced for 1 h by the addition of 0  $\mu$ M, 10  $\mu$ M, 30  $\mu$ M, 100  $\mu$ M, 300  $\mu$ M and 1000  $\mu$ M  $CuSO_4$  (lanes 1–6). (B) Quantification of the data shown in A. ●, CopB from the genomic wild-type promoter; ○, CopB665 from plasmid  $P_{WT}$ ; ▲, CopB665 from plasmid  $P_A$ ; △, CopB665 from plasmid  $P_{AB}$ . (C) Induction of CopB665 from  $P_{AB}$  in the low copper concentration range. ○, copper(I); ●, copper(II). Details of the methods are described under Materials and Methods.

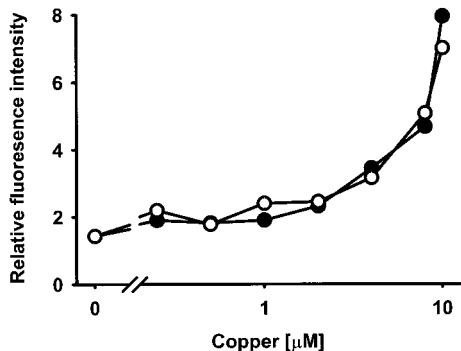
dosage effects (16). The promoter mutation A-61T (start of translation = 1) had previously been shown to impede the interaction of CopY with the operon-distal DNA binding site *in vitro*. When CopB665 was placed under the control of the A-61T mutant promoter on a plasmid ( $P_A$ ), induction kinetics by copper were similar to those of the wild-type promoter, except for a higher induction level at 1 mM copper. In this and all further experiments, the A-61T and the A-30T single mutations exhibited essentially the same properties as the wild-type promoter and are therefore not shown in subsequent experiments. In contrast to the single mutations, the plasmid-borne A-61T/A-30T double mutant promoter ( $P_{AB}$ ) was fully induced by only 10  $\mu$ M copper, conditions that did not significantly induce transcription from  $P_{WT}$  or  $P_A$ . The reproducible depression of the induction from  $P_{AB}$  at 100 to 300  $\mu$ M copper remains unexplained at present.

Taken together, it appears that one mutated CopY binding site on the promoter does not strongly disturb the control of expression, except under very high copper conditions. Mutation of both CopY binding sites in  $P_{AB}$  led to hyperinduction by copper. This is in agreement with previous *in vitro* studies on CopY-DNA interaction by band shift assays (3). In these experiments, the affinity of CopY for  $P_A$  or  $P_B$  was only lowered two- to three-fold, while essentially no CopY

binding to  $P_{AB}$  could be observed, suggesting cooperativity in CopY binding to the two sites on the promoter. *In vivo*, the basal level of expression from  $P_{AB}$  is higher than in the wild-type, but can still be strongly induced by copper. Thus, *in vivo* there is still binding of CopY to the double mutant promoter and this binding can be abolished by copper, albeit more easily than binding to wild-type promoter.

Figure 2C depicts the gradual induction of the  $P_{AB}$  promoter by low concentrations of copper(I) or copper(II). In this as well as all other experiments reported here, there was no significant difference between the effects of copper(I) or copper(II), suggesting that both ions communicate with the cytoplasm. Also, the same results were obtained whether copper(I) was generated by reduction with 1 mM ascorbate or added as a Cu(I)-acetonitrile complex in the presence of 2% acetonitrile (not shown, 17).

In wild-type cells, the intracellular copper concentration is regulated by the copper homeostatic system and the effect of externally added copper on the cytoplasmic concentration is unknown. We therefore employed a  $\Delta copAB$  mutant strain, B1, that is deficient in the import as well as the export copper ATPase and that is very sensitive to copper (8). Figure 3 shows that in B1, induction of the  $P_{AB}$  double mutant promoter was less responsive to induction by  $Cu^+$  or  $Cu^{2+}$  than in wild-



**FIG. 3.** Copper induction of CopB665 from  $P_{AB}$  in the  $\Delta copAB$  double mutant B1.  $\circ$ , Induction by copper (I);  $\bullet$ , induction by copper(II). Expression levels were determined as in Fig. 2.

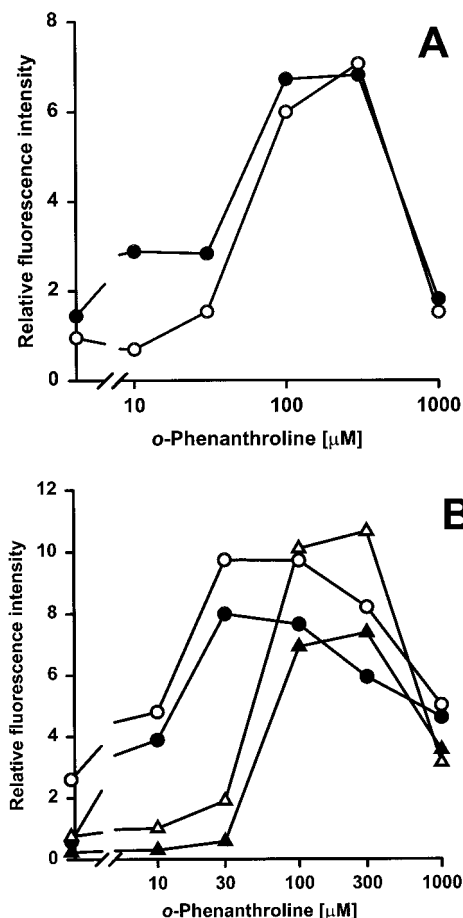
type cells. This suggests that copper import results in a higher cytoplasmic copper concentration in wild-type *E. hirae* compared to B1. No induction from wild-type or singly mutated promoters could be observed in B1 due to the low copper tolerance of this strain (not shown).

**Induction of the *cop* promoter by copper(II) chelators.** It had been shown previously that the *cop* operon is induced by the chelator *o*-phenanthroline and that this induction was a consequence of copper rather than iron limitation (8). Figure 4A shows that CopB expression from the genomic wild-type *cop* promoter was half-maximally induced at approximately 50  $\mu M$  *o*-phenanthroline and that there were no significant *trans* or gene dosage effects by the presence of reporter plasmids. As apparent from Fig. 4B, the *o*-phenanthroline induction kinetics of CopB665 from plasmid-borne  $P_{WT}$  and  $P_{AB}$  were essentially the same and thus differed dramatically from induction by copper, which hyperinduced  $P_{AB}$ . In the  $\Delta copAB$  strain B1, *o*-phenanthroline induction of  $P_{WT}$  and  $P_{AB}$  also behaved similarly, but half-maximal induction was shifted to 10  $\mu M$ . So analogous to induction by copper, induction by *o*-phenanthroline was shifted to lower concentrations in B1 compared to wild-type cells, which again must be ascribed to the copper homeostatic control in the wild-type. Essentially the same results were obtained using tetrathiomolybdate as a copper chelator (not shown). That wild-type and double mutant promoters responded similarly to induction by copper(II) chelators suggests that induction by copper complexation does not work *via* release of CopY from the promoter, which would be expected to lead to hyperinduction of the double mutant promoter. This agrees with *in vitro* band shift experiments, where it had been shown that copper, but not *o*-phenanthroline, released CopY from the DNA binding site (3), D. Strausak, unpublished observation).

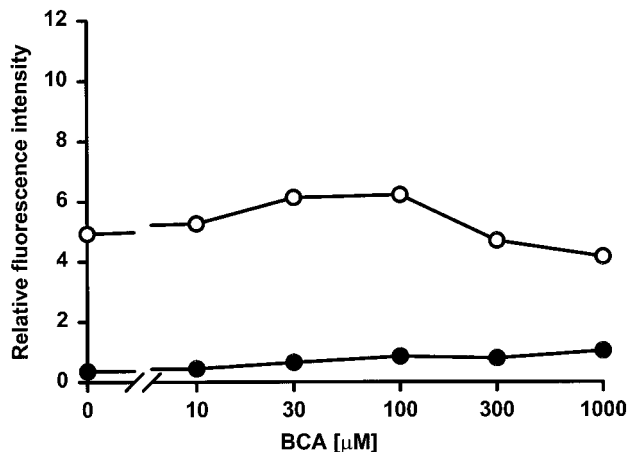
In contrast to *o*-phenanthroline and tetrathiomolybdate, which complex copper(II), the specific copper(I)

chelator bicinchoninic acid (BCA) did not induce expression from either wild-type or mutant *cop* promoters (Fig. 5). The same was observed for the copper(I) chelator bathocuproin disulfonate (not shown).

**Identification and properties of an extracellular copper reductase.** The failure of the copper(I) chelators to induce the *cop* operon was unexpected. It led to the hypothesis that an extracellular copper reductase generates copper(I) in an occluded space, not accessible to chelators, such as the periplasm. Figure 6 demonstrates the presence of an extracellular copper reductase activity in *E. hirae*. Energy (glucose) dependent reduction of copper(II) to copper(I) by whole cells could be detected in a photometric assay with BCA (18). When *E. hirae* was grown in normal media, copper reductase activity was  $0.14 \pm 0.03$  nmol/min/ $10^8$  cells. This activity was reduced to approximately 50% if the cells were grown in the presence of 40  $\mu M$  copper(II) or 300  $\mu M$  *o*-phenanthroline and thus did not appear to be



**FIG. 4.** *o*-Phenanthroline induction in wild-type and B1 mutant. (A) Induction of genomic CopB in wild-type cells ( $\bullet$ ) and in wild-type cells containing also a plasmid-borne *cop* operon ( $\circ$ ). (B) Induction of CopB665 from plasmid  $P_{WT}$  ( $\blacktriangle$ ) and  $P_{AB}$  ( $\triangle$ ) in wild-type cells and from plasmid  $P_{WT}$  ( $\bullet$ ) and  $P_{AB}$  ( $\circ$ ) in the  $\Delta copAB$  mutant B1. Details of the procedure were as in Fig. 2.



**FIG. 5.** Induction of CopB665 by copper chelation. Induction of CopB665 from plasmid-borne P<sub>WT</sub> (●) and P<sub>AB</sub> (○) in wild-type cells by the copper(I) chelator BCA. Details of the procedure were as in Fig. 2.

strongly regulated. Copper reduction was very sensitive to inhibition by mercury(II), with an  $I_{50}$  of 1  $\mu\text{M}$  (Fig. 6B). Silver(I) which has a similar ionic radius to copper(I) inhibited with an  $I_{50}$  of 50  $\mu\text{M}$ . Nickel(II), tin(II), cadmium(II) and platinum(I) at 100  $\mu\text{M}$  had no significant inhibitory effects. Since platinum(II) is an inhibitor of Fre1p, a yeast iron and copper reductase (estimated  $I_{50}$  for Pt(II) = 50  $\mu\text{M}$ , ref. 14), its effect at higher concentrations was also investigated. Pt(II) inhibited the *E. hirae* copper reductases activity by 50% at the maximally attainable concentration of 1 mM, indicating that the *E. hirae* enzyme differs from Fre1p in its biochemical properties. This *E. hirae* reductase activity is, to our knowledge, the first bacterial copper reductase to be described. An iron reductase activity that amounted to 70% of the copper reductase activity could also be detected. Since it was fully inhibited by 100  $\mu\text{M}$  platinum(II), it was clearly due to a different catalytic activity (not shown).

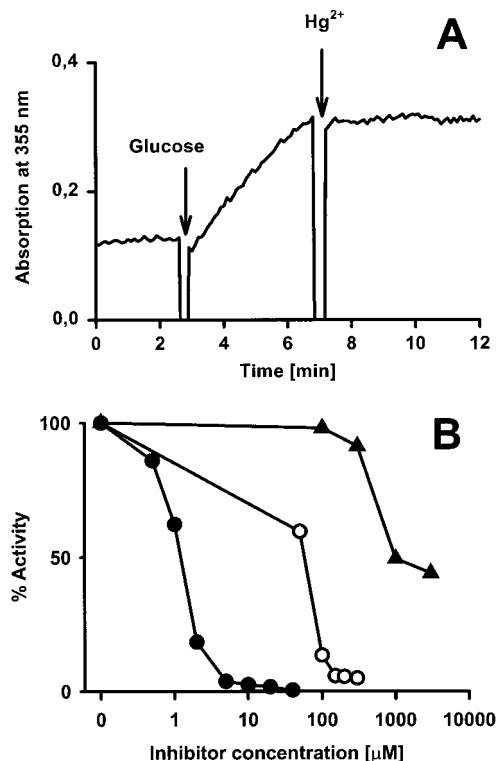
*Effect of a copper reductase mutant on the induction of the cop operon.* In light of the presence of an extracellular copper reductase activity, it became of interest to look at the influence of copper reduction on the regulation of the *cop* operon. With a colorimetric plate assay, several mutants with reduced extracellular copper reductase activity were isolated from mutagenized cells. Mutant C4 and several other mutants that were identified had a copper reductase activity that was reduced by 70%. However, no mutants completely deficient in copper reductase could be isolated. In mutant C4, induction of wild-type and mutant *cop* promoters by either copper(I) or copper(II) did not differ significantly from that observed in wild-type cells (not shown).

Taken together, our data show that both, copper(I) and copper(II) were equally effective in inducing the

*cop* operon. One intact CopY binding site was sufficient for effective repression. Mutation of both CopY binding sites facilitated induction by copper and did so to the same extent for copper(I) and copper(II). The induction kinetics by copper(I) or copper(II) were not influenced by reduced extracellular copper reductase activity. In contrast to induction by copper, induction by copper chelators was not influenced by promoter mutations that impair the interaction of CopY with the promoter, indicating that this induction occurs by a different mechanism.

## DISCUSSION

The bi-phasic induction of the *cop* operon of *E. hirae* by excess as well as limiting copper reflects its dual role in copper uptake and copper secretion (9). This co-regulation of the import and the export copper ATPase may be a safety mechanism: a sudden change from copper limiting to copper excess conditions could poison the cell if the import pump is induced, but no export route is available. The mechanism of this regulation is not fully understood. We thus studied, *in vivo*, aspects of wild-type and mutant *cop* promoter regula-



**FIG. 6.** Copper reductase activity of *E. hirae* wild-type cells. (A) The formation of a copper(I) bicinchoninic acid complex catalyzed by whole cells was followed spectrophotometrically as described under Experimental Procedures. Where indicated, 1% glucose or 2  $\mu\text{M}$   $\text{HgCl}_2$  were added. (B) Inhibition of copper reductase activity by  $\text{Hg}^{2+}$  (●),  $\text{Ag}^+$  (○), and  $\text{Pt}^{2+}$  (▲).

tion by copper(I), copper(II), and copper chelators, and the role of extracellular copper reduction.

It had previously been shown *in vitro* that CopY binds to two sites of the *cop* promoter and that copper(I) dissociates CopY-DNA complexes with an apparent half-binding constant of 20  $\mu\text{M}$  (3). In the current study, we assessed induction *in vivo* with a reporter protein on a plasmid-borne system. In wild-type cells, half-maximal induction of the wild-type promoter was observed at approximately 1 mM copper. Cytoplasmic copper is of course controlled by the copper homeostatic system and the concentration is unknown. Mutant strain B1, unable to control cytoplasmic copper due to knock-out of the two copper pumps, proved to be too sensitive to copper to observe induction of the wild type *cop* promoter while double-mutant promoters were induced by the low copper levels applicable to this strain.

The double mutant promoter, A-61T/A-30T was approximately 1000-fold more susceptible to copper induction than the wild-type promoter. Each single mutation, A-61T and A-30T, respectively, showed induction kinetics resembling those of the wild-type, suggesting that one intact repressor binding site is sufficient for the control of transcription under our experimental conditions. That the induction mechanism of the *cop* operon by copper is *via* release of CopY from the DNA is corroborated the observation that in a CopY knock-out strain, the *cop* operon is fully induced under all conditions (9).

The induction of the double mutant promoter by micromolar added copper raises the issue of the meaning of copper concentrations. The growth media had a basal copper content of 10  $\mu\text{M}$  as assessed by atomic absorption spectroscopy. Yet, this copper did not lead to induction. However, addition of even sub-micromolar concentrations of copper induced the double mutant promoter P<sub>AB</sub>. This suggests that the endogenous media copper is tightly bound to media components and not bio-available, while added copper is biologically active. Since copper will always be complexed by components of the system in an unknown way, the issue of biologically active copper concentrations remains unresolved at present and 'concentration' as used here must be regarded as a functional term only.

Interestingly, in the B1 mutant strain deficient in the two copper ATPases and thus unable to control cytoplasmic copper levels, induction of the double mutant promoter was less responsive to copper than in wild-type cells. Again, copper(I) and copper(II) exhibited the same induction kinetics and must thus both be able to access the cytoplasm with similar efficiency. The aggravated response to copper in the wild-type compared to B1 indicates that wild-type cells accumulate copper even at 1  $\mu\text{M}$  extracellular copper. Increases in the zinc concentrations required for induction of zinc transport genes have similarly been observed in zinc transport mutants of *B. subtilis* and yeast (19, 20).

In contrast to the nearly thousand fold better copper induction of the double mutant promoter compared to the wild-type, no such difference was apparent for induction by *o*-phenanthroline. This observation suggests that induction of the *cop* operon by copper deficiency occurs by a different mechanism than induction by excess copper. Data presented previously and in this report support the following model of the regulation of the *cop* operon: In normal growth media, CopY is bound to the *cop* promoter and transcription of all *cop* genes is repressed. If cytoplasmic copper rises, CopY is released from the promoter and the *cop* genes are induced. This transfer of copper to CopY is accomplished by the CopZ copper chaperone (4, 9). If copper gets limiting in the cytoplasm, CopY is not released from the DNA and transcription of the *cop* operon is induced by a different mechanism. Although we cannot exclude that CopY or CopZ has a dual function in induction by excess as well as limiting copper, we favor a mechanism involving other components, such as a sigma factor or a stress response element. However, the fact that induction by *o*-phenanthroline was shifted to lower concentrations in mutant cells deficient in copper homeostasis suggests that *o*-phenanthroline also signals its effect on *cop* operon expression *via* copper. This issue will be addressed in future studies.

Copper circulation in *E. hirae* may thus proceed as follows: CopA catalyzes the uptake of copper(I). Since copper(I) ions are essentially insoluble in free form at physiological pH, they are generated near the plasma membrane by the copper reductase and the necessary copper is drawn from this pool. Because of this reductase activity, it is irrelevant whether copper(I) or copper(II) is supplied to the cell. Copper(II) chelators can effectively remove all the substrate for the copper reductase and simulate copper deficiency, leading to induction of the *cop* operon. Copper(I) chelators, on the other hand, cannot efficiently access the copper(I) that is generated near the plasma membrane, and thus do not affect intracellular copper and consequently the regulation of the *cop* operon. All the copper reductase mutants that were isolated by chemical mutagenesis had a residual activity of around 30%, precluding the unequivocal assignment of a role to this enzyme. This could be a shortcoming of chemical mutagenesis or be indicative of two copper reductase activities. To inhibit the copper reductase with mercury and to look at the effect on regulation was attempted, but Hg<sup>2+</sup> proved too toxic to *E. hirae* to conduct this experiment. Current work is thus aimed at employing transposon mutagenesis to inactivate and clone the copper reductase of *E. hirae*.

The only other bacterial copper regulatory system that has been studied in some detail is the plasmid-borne copper resistance operon of *Pseudomonas syringae*. This operon is metalloregulated by a two-component regulatory system involving CopR and

CopS (21). Copper triggers the membrane bound copper sensor CopS to phosphorylate CopR, which then acts as a transcription factor to stimulates expression of the *P. syringae cop* operon. Homologues of CopR and CopS, called PcoR and PcoS, have also been identified on an *E. coli* plasmid, but their function has not been investigated in great detail. Although two-component sensor/responder phosphokinase regulatory systems are a common theme in bacterial regulation, the regulation of the *E. hirae cop* operon clearly represents a different regulatory mechanism. From genome sequencing projects we know that most, if not all bacteria, have enzymes related to the CopA and/or CopB copper ATPases of *E. hirae*. It will be interesting to learn, in the future, how they are regulated in comparison to the *cop* operon of *E. hirae*.

#### ACKNOWLEDGMENTS

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