Measurement of cytoplasmic copper, silver, and gold with a *lux* biosensor shows copper and silver, but not gold, efflux by the CopA ATPase of *Escherichia coli*

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Abstract Copper, silver, gold and other heavy metals are potentially toxic to cells. Copper is also essential and cellular levels must be carefully controlled. In contrast, there is no known biological role for silver or gold and they have not been recognized as metals that are under homeostatic control. Using a luminescent biosensor based of the *Vibrio fischeri lux* gene cluster under the control of the *Escherichia coli copA* promoter/CueR metal-responsive regulator, we could show that in *E. coli*, cytoplasmic copper and silver, but not gold, are regulated by the CopA ATPase, the major copper efflux pump.

Key words: CPx-type ATPase; CopA; CueR; Copper homeostasis; Biosensor; Lux; Silver; Gold

1. Introduction

Copper is essential for life because of its distinct oxidation states and useful redox potentials of 200–800 mV when protein-bound. On the other hand, copper ions are very reactive and can take part in Fenton-type reactions, the products of which can cause oxidative damage [1]. This has necessitated the evolution of tight homeostatic control systems. In the last few years, great progress has been made in understanding the copper homeostasis in both prokaryotes and eukaryotes (see [2–7] for review). In *Escherichia coli*, at least 10 chromosomal genes involved in two distinct copper homeostasis systems have been identified [7–14].

The copper-transporting ATPase, CopA, is the central component of copper homeostasis in *E. coli*. This enzyme belongs to the subclass of heavy metal-transporting CPx-type ATPases [15]. It was observed that the expression of CopA increased upon addition of copper and silver [8]. Further, it was shown that transcription from the *copA* promoter was activated by copper, silver, and gold via the CueR activator [10,13,16]. While the transport of copper(I) was demonstrated with everted native membrane vesicles which accumulated ^{64}Cu [8], there is currently no evidence for the transport of other ions by CopA. This raised the question whether only the transcription of *copA* is activated by silver and gold, or whether CopA can also transport these ions.

In an attempt to assess cytoplasmic copper, silver, and gold in *E. coli*, we constructed a biosensor based on the *Vibrio fischeri lux* gene cluster under the control of the *E. coli copA*/CueR promoter/activator system. By measuring the luminescence, we could show the variation in cytoplasmic copper, silver and gold levels under different ambient metal concentrations, but also between wild-type and mutant cells defective in the CopA heavy metal extrusion system. Using the biosensor, it could be shown that in cells deficient in CopA, both cytoplasmic copper and silver, but not gold, were increased compared to wild-type cells. This provides the first evidence for homeostatic control of silver by CopA in *E. coli*. Gold also induced the biosensor, but cytoplasmic levels did not appear to be regulated.

2. Materials and methods

2.1. Materials

Chelex 100, 100–200 mesh, was purchased from Bio-Rad. All other chemicals were from Merck (Darmstadt, Germany), or from Sigma and were of analytical grade. The *E. coli* wild-type strain W3110 and the *copA* knockout strain DW3110 have been described previously [7]. For growth experiments, logarithmically growing cells frozen at −70°C in 17% glycerol (frozen log cells) were used for inoculation.

2.2. Construction of a lux reporter plasmid

The reporter plasmid was constructed by inserting the *copA* promoter into pUCD615, containing the *lux* gene cluster of *V. fischeri* without a promoter [17]. The CopA promoter was constructed by annealing the two 54-mer oligonucleotides Psynt/1, 5′-*GAT C*CT TCT TGA CCT TCC CCT TGC TGG AAG GTT TAA CCT TTA TCA CAG CCA GT*G*, and Psynt/2, 5′-*AAT TC*A CTG GCT GTG ATA AAG GTT AAA CCT TCC AGC AAG GGG AAG GTC AAG AA*G* as described [10]. Nucleotides added for cloning are italicized. The promoter DNA was ligated into pUCD615, digested with *Eco*RI and *Bam*HI, creating plasmid pUA615, in which the CopA promoter drives the expression of the *lux* genes.

2.3. Measurement of lux induction

For induction studies, the *E. coli* wild-type strain W3110 and the *copA* knockout strain DW3110 [7] were transformed with pUA615 and grown overnight at 37°C in the presence of 50 μg/ml of ampicillin in modified SM9 medium (containing per liter: 1.5 g Na_2HPO_4, 0.75 g KH_2PO_4, 0.5 g NH_4Cl, 1 mM $MgSO_4$, 0.6 g casein hydrolysate, 0.4 g glucose, 2 mg thiamine). Fresh cultures were inoculated with the overnight cultures and grown to mid-log phase ($OD_{550} \approx 0.1$) in modified SM9 medium without antibiotics and treated with Chelex 100 resin as follows: 1 g of Chelex 100 was added to 1 l of medium, swirled for 1 h, and the Chelex removed by filtration. Culture aliquots of 300 μl were induced with $CuSO_4$ or $AgNO_3$ in triplicate in microtiter plates for the times specified in Section 3. For the induction by $HAuCl_4$, cells were grown in LB medium and luminescence was measured after 2 h. Emitted light was measured with an LAS-1000 CCD camera (Fuji

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Photo Film, Japan) for 5 min. The images were integrated with the AIDA software (Raytest Isotopenmessgeräte, Straubenhardt, Germany). Induction by gold was measured by the same procedure, except that LB medium was used.

2.4. Determination of growth rates

For growth measurements, modified SM9 medium was inoculated with log cells, stored frozen in 17% glycerol at −80°C, at a density of 4×10^7 cfu/ml. Cultures were grown aerobically at 37°C. $AgNO_3$ at the concentrations specified in Section 3 was added 1 h after inoculation and growth was followed by measuring the absorption at 550 nm. Growth rate was expressed as 1/doubling time.

3. Results

3.1. Time course of bioluminescence response

E. coli cells containing the biosensor plasmid pUA615 exhibited luminescence in response to externally added $CuSO_4$, $AgNO_3$, or $HAuCl_4$. To assess the stability of the luminescence signal over time, luminescence was measured at different times following addition of heavy metal ions to the medium. Fig. 1 shows the time course of the induction of luminescence in wild-type and a Δ*copA* strain by different copper concentrations. Luminescence was maximal 1–1.5 h after induction in both strains and at 10 and 60 μM ambient copper. Luminescence remained at 50–90% of this level up to 4 h after induction, except in the Δ*copA* strain in 60 μM copper, where luminescence started to decline rapidly 1.5 h after induction. This was due to growth inhibition of the Δ*copA* strain by 60 μM copper. Essentially the same induction kinetics were observed for silver and gold (not shown). If broth was used instead of semi-synthetic medium, qualitatively similar results were observed, but much higher metal ion concentrations were required to obtain maximal induction (not shown). This was presumably due to the absorption of the heavy metal ions to components of complex medium. Based on the maximal induction of luminescence 1 h after induction, a 1 h induction time was used in subsequent experiments.

3.2. Concentration dependence of the bioluminescence response

The different responses of wild-type and Δ*copA* cells to copper and silver described in Fig. 1 were analyzed in more

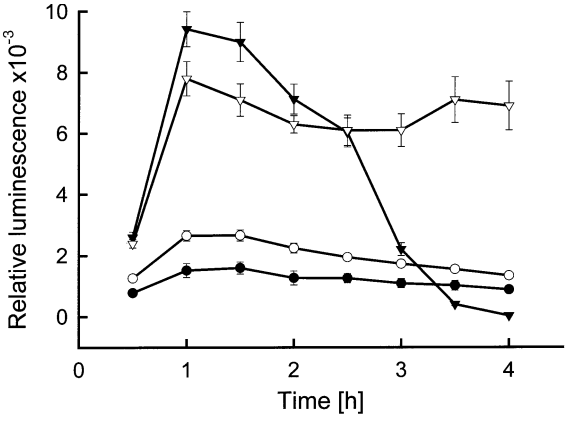

Fig. 1. Time course of luminescence induction. Luminescence was measured in wild-type *E. coli* or a Δ*copA* strain, transformed with the biosensor plasmid pUA615, at different times after addition of heavy metal ions to the medium. ●, wild-type in 10 μM Cu^{2+}; ○, wild-type in 60 μM Cu^{2+}; △, Δ*copA* strain in 10 μM Cu^{2+}; ▼, Δ*copA* strain in 60 μM Cu^{2+}. Details of the procedure are described in Section 2.

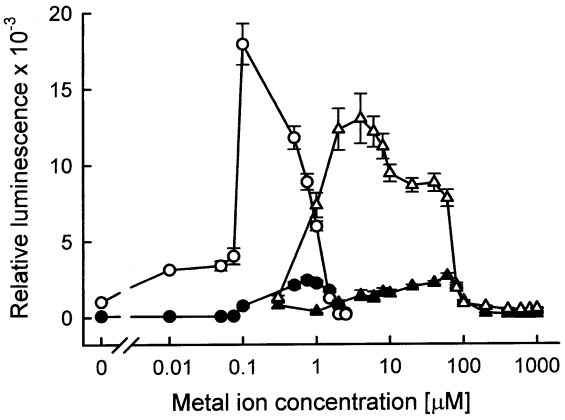

Fig. 2. Induction of luminescence by silver and copper. Wild-type and Δ*copA* cells were exposed to different concentrations of Cu^{2+} and Ag^+ in modified semi-synthetic medium for 1 h and the luminescence response was measured. ▲, wild-type, Cu^{2+}; ●, wild-type, Ag^+; △, Δ*copA*, Cu^{2+}; ○, Δ*copA*, Ag^+. Measurements were conducted in triplicate and three independent experiments were averaged. The error bars indicate the S.D. Other details are describe in Section 2.

detail. Titration of silver and copper over a concentration range of 10^5 revealed two interesting points. First, for maximal luminescence in wild-type, 80-fold higher concentrations of copper than silver had to be employed (Fig. 2; 0.75 μM Ag^+ versus 60 μM Cu^{2+}). Secondly, in the Δ*copA* strain, an eight-fold lower concentration of silver and a 15-fold lower concentration of copper, compared to wild-type, was required to elicit maximal luminescence (0.1 μM Ag^+, 4 μM Cu^{2+}). At the same time, overall luminescence intensities were enhanced five- to seven-fold. The more sensitive and more pronounced response of luminescence to copper and silver in the Δ*copA* strain is supposedly due to increased intracellular levels of these ions. This suggests that the homeostatic control of both copper and silver is impaired in the Δ*copA* strain. The only difference between the two strains is the expression of a functional CopA ATPase in the wild-type, which is lacking in the Δ*copA* strain. The CopA ATPase thus appears to efflux not only copper ions, but also silver ions. Since the expression of the CopA ATPase is also regulated by silver via the CueR activator [10], this appears to represent a true homeostatic control system for silver.

3.3. Induction of bioluminescence on solid medium

The exceptional sensitivity of the Δ*copA* strain to copper and the enhanced luminescence response from the biosensor could also be shown in a more tangible way. On a plate with synthetic medium, wild-type cells were plated on one half of the plate and Δ*copA* cells on the other. A copper coin placed in the center of the plate immediately after plating released sufficient amounts of copper to generate a zone of luminescence, which was considerably more intense in the Δ*copA* strain (Fig. 3). Luminescence was confined to a zone which apparently featured the optimal copper concentration.

3.4. Growth inhibition by silver

If there is homeostatic control of silver by CopA, wild-type cells would be expected to be more resistant to silver than a Δ*copA* mutant strain. This could indeed be observed. Fig. 4 shows that wild-type cells were more silver-resistant than

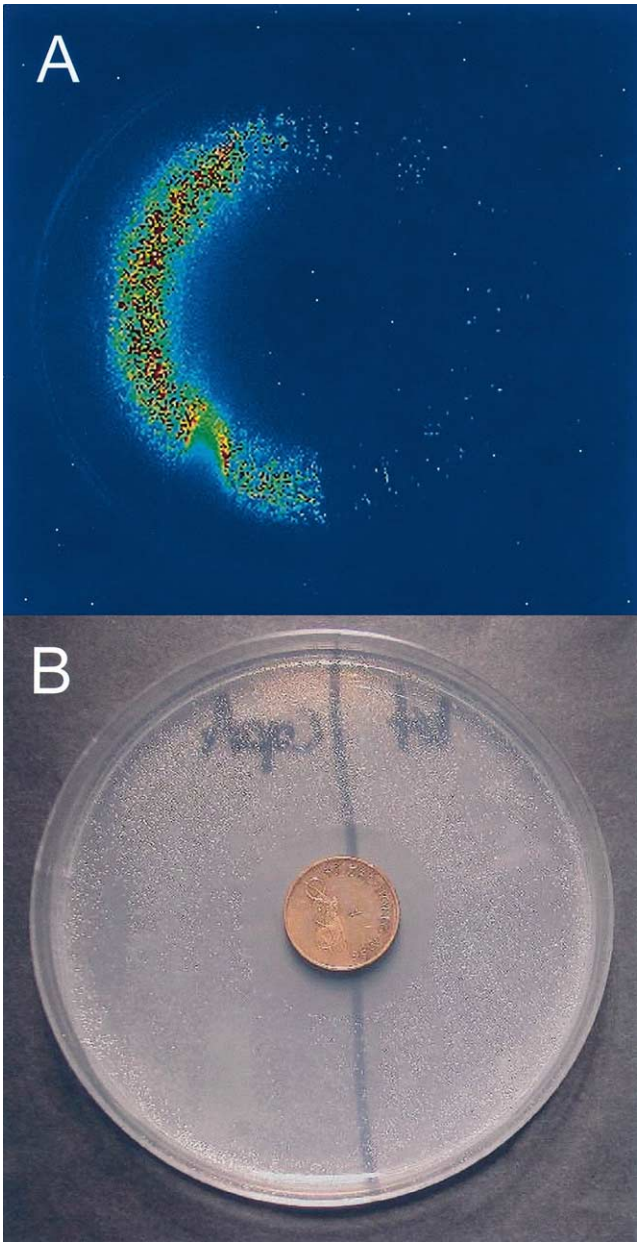

Fig. 3. Induction of luminescence by a copper coin. A Norwegian copper 50-øre coin was placed in the center of an agar plate with modified semi-synthetic medium on which wild-type *E. coli* (right half) and Δ*copA* cells (left half) had been plated. After incubation for 14 h, the plate was photographed in white light (B) and luminescence was recorded and displayed in false colors (A).

Δ*copA* cells. The wild-type still grew at half the maximal growth rate in 0.2 μM Ag^+, which fully inhibited growth of the Δ*copA* strain. To see this difference in silver resistance between wild-type and Δ*copA* cells required the use of the NaCl-free, specially formulated semi-synthetic medium used here.

3.5. Induction of bioluminescence by gold

It had previously been shown that the *copA* promoter/CueR activator system used here for the control of the *lux* operon also responds to gold [16]. To test whether this activation by gold is fortuitous or is also part of homeostatic control by CopA, the response of the biosensor to Au^{3+} was assessed. As

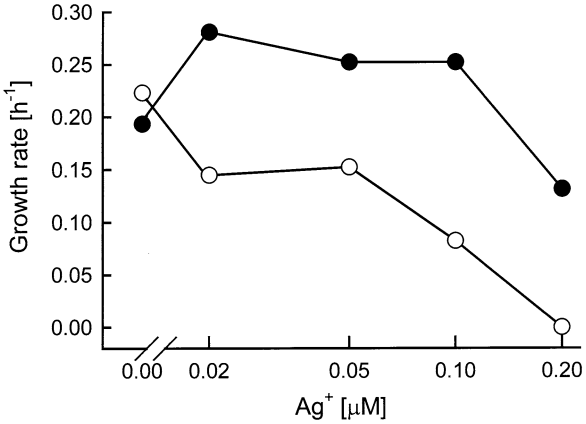

Fig. 4. Growth inhibition of wild-type and Δ*copA* cells by silver. Cells were grown in modified semi-synthetic medium and growth was followed by measuring the absorption at 550 nm. Growth rates were determined from the slopes of individual growth curves (mean of two), and expressed as 1/doubling time. Other details are as described in Section 2. ●, wild-type; ○, Δ*copA*.

expected, the biosensor responded to gold, with maximal activation observed in 30–40 μM gold (Fig. 5). Importantly, however, there was no significant difference in sensitivity and intensity of the luminescence response between wild-type and Δ*copA* cells. This argues against a participation of the CopA ATPase in gold homeostasis. The finding also underlines that the observed differences in copper and silver responses between wild-type and Δ*copA* cells are not due to strain differences, but most likely to differences in cytoplasmic heavy metal ion concentrations.

4. Discussion

A major problem in assessing homeostatic control of inorganic ions is the measurement of apparent cytoplasmic concentrations. This is particularly challenging for heavy metal ions such as copper, silver, gold, mercury, or cadmium, as these avidly bind to cellular constituents. We here used the *lux* gene cluster of *V. fischeri*, placed under the control of the *E. coli copA* promoter/CueR activator, to obtain in vivo mea-

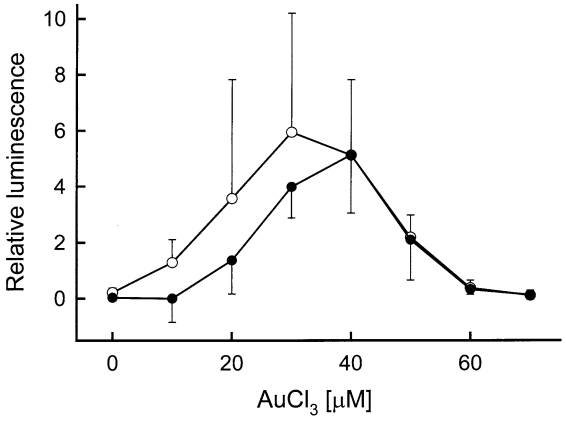

Fig. 5. Induction of luminescence by gold. Wild-type and Δ*copA* cells were exposed to different concentrations of Au^{3+} and the luminescence response was measured. Measurements were conducted in triplicate and three independent experiments were averaged. The error bars indicate the S.D. Other details are described in Section 2. ●, wild-type; ○, Δ*copA*.

surements of cytoplasmic copper, silver, and gold levels. We could show that in cells deficient in the major copper efflux pump, CopA, maximal luminescence was elicited by 15-fold lower concentrations of ambient copper than in the wild-type. Similarly, eight-fold lower concentrations of extracellular silver were required for maximal luminescence in a Δ*copA* strain. Luminescence was also induced by gold, but without a significant difference between the wild-type and a Δ*copA* strain. These observations show on one hand that the biosensor can be employed to assess apparent cytoplasmic copper, silver, and gold concentrations and, on the other hand, that there is homeostatic control of copper and silver, but not gold, by the CopA ATPase. Silver efflux by CopA was corroborated by demonstrating increased silver resistance of wild-type compared the Δ*copA* strain. Silver or gold resistance mediated by CopA could previously not be observed [8,16]. This may be due to the narrow range of resistance, combined with its variation with experimental conditions. Medium components can complex heavy metal ions and we used a specially formulated semi-synthetic medium for these experiments.

Ag^+ transport by CPx-type Cu^+ ATPases may be a general phenomenon. Solioz and Odermatt demonstrated Ag^+ transport by the related CopB copper efflux ATPase of *Enterococcus hirae* [18]. In isolated membrane vesicles, CopB pumped Ag^+ and Cu^+ at similar rates and with similar affinities. Putative silver pumps were also identified on a plasmid isolated from silver-resistant *Salmonella*. Nine open reading frames were implicated in the resistance mechanism [19]. These genes encode, among others, a putative silver CPx-type ATPase, SilP, and a three-component silver/proton antiporter, SilCBA, with sequence similarity to the Czc (cadmium, zinc, cobalt) resistance system of *Alcaligenes* (now *Ralstonia*) and the related YlcBCD-YbdE system of *E. coli*, which has been shown to confer silver resistance [20,21]. Other observations also suggest that copper ATPases can pump silver: (i) CopA of *Archaeoglobus fulgidus* was shown to be more strongly activated by silver(I) than by copper(I), although the enzyme exhibited a higher affinity for copper(I) [22], (ii) in the fungus *Candida albicans*, the CPx-type ATPase encoded by CRD1 was shown to produce cellular copper and silver resistance [23], and (iii) *E. coli* expressing the Bxa1 CPx-type ATPase from *Oscillatoria brevis* was more silver-resistant than wild-type cells [24]. Due to the current, apparently world-wide, unavailability of radioactive silver, transport of this ion could not be demonstrated directly for most of these systems.

What is the biological significance of silver efflux by CopA? The recent discovery of the oldest known microfossils in deep-sea volcanic rock suggests that hydrothermal vents may be the site of evolution of the first living systems on earth [25]. The hot, acidic seawater encountered at these vents is rich in heavy metal ions dissolved from the volcanic rock [26] and resistance to these metal ions may have been an evolutionary priority for early life forms. High evolutionary conservation of heavy metal ATPases across phyla and the divergence of heavy metal and non-heavy metal ATPases before the division into eukaryotic and prokaryotic cells support this view [15,27]. Also, the broad ion specificity of CPx-type ATPases suggests that they evolved primarily for the detoxification of the cellular cytoplasm.

In summary, using a biosensor which allows real-time, in vivo monitoring of apparent cytoplasmic copper, silver, and gold concentrations, we showed that CopA of *E. coli* can control cytoplasmic silver, in addition to copper. The biosensor described here should be applicable to a wider range of problems in heavy metal homeostasis research.

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