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Response to excess copper in the hyperthermophile *Sulfolobus solfataricus* strain 98/2

Aramis A. Villafane, Yekaterina Voskoboynik [1], Mariola Cuebas [†], Ilona Ruhl, Elisabetta Bini [*]

Department of Biochemistry and Microbiology, and Biotechnology Center for Agriculture and the Environment, Rutgers, The State University of New Jersey, New Brunswick, NJ 08901-8525, USA

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ABSTRACT

Copper is an essential micronutrient, but toxic in excess. *Sulfolobus solfataricus* cells have the ability to adapt to fluctuations of copper levels in their external environment. To better understand the molecular mechanism behind the organismal response to copper, the expression of the cluster of genes *copRTA*, which encodes the copper-responsive transcriptional regulator CopR, the copper-binding protein CopT, and CopA, has been investigated and the whole operon has been shown to be cotranscribed at low levels from the *copR* promoter under all conditions, whereas increased transcription from the *copTA* promoter occurs in the presence of excess copper. Furthermore, the expression of the copper-transporting ATPase CopA over a 27-h interval has been monitored by quantitative real-time RT-PCR and compared to the pattern of cellular copper accumulation, as determined in a parallel analysis by Inductively Coupled Plasma Optical Emission spectrometry (ICP-OES). The results provide the basis for a model of the molecular mechanisms of copper homeostasis in *Sulfolobus*, which relies on copper efflux and sequestration.

Introduction

Copper is a transition metal and an important trace element because of the essential role it plays in a range of biological processes. In contrast, the occurrence of copper levels beyond the physiological range causes serious damage to all molecular components. Studies on yeast have led to the proposition that virtually no free copper ions are present in the cell under normal conditions [1]. The response of cells to copper excess/deficiency is accomplished through the interplay of copper-binding proteins, copper-responsive regulators, transporters for the efflux and uptake of copper, and copper-requiring enzymes. Genetic determinants of copper homeostasis have been described for several bacterial species [2–7]. In particular, the various components of the *Enterococcus irae* and *Escherichia coli* copper-homeostasis systems, their regulation, and interactions have been thoroughly studied [2,8]. Many sequenced archaeal genomes encode homologs of Cu (I) and Cu (II) transporting ATPases [9]. However, investigations of the response in archaea to changes of copper levels are still limited. Structural studies of individual functional domains of the Cu (I)-transporting ATPase CopA in *Archeoglobus fulgidus* have provided useful insights into its activities and functions [10–14]. *A. fulgidus* also possesses a Cu(II)-transporting ATPase, CopB, that has been biochemically characterized [15]. The transcriptional analysis of a *cop* locus responsible for survival in the presence of copper has been reported in the extreme acidophilic archaeon "*Ferroplasma acidarmanus*" strain Fer1, where cotranscription of genes encoding the copper-binding protein CopZ and the putative copper-transporting ATPase CopB was shown to increase in response to Cu (II) [16]. An interesting mechanism for copper detoxification has been described in *Sulfolobus metallicus*, which is based on sequestration by organic phosphate, possibly followed by active efflux of the metal-phosphate complex [17]. The *Sulfolobus solfataricus* genome encodes a *cop* locus, which includes the three open reading frames (ORFs) Sso2651, Sso2652, and Sso10823, encoding the CopA ATPase, a copper-responsive regulator, and a putative copper-binding protein, respectively [18]. Cotranscription of Sso2651 and Sso10823 has been reported to specifically increase in the presence of copper, while the copper-responsive regulator binds sequences surrounding the putative *copA* promoter in *S. solfataricus* strain P2 [19]. In this study, the response of *S. solfataricus* to copper has been further investigated in the strain 98/2. The selection of the genetically tractable strain 98/2 [20] will expand the scope of analyses aimed to the elucidation of archaeal interactions with copper. To gain better insights into the *Sulfolobus* response to copper levels, the transcription of the three genes of the *copRTA* operon has been examined under different conditions and in a time course experiment, and the changes in the amount of copper associated with

* Corresponding author. Address: Department of Biochemistry and Microbiology, Rutgers University, 76 Lipman Drive, New Brunswick, NJ 08901-8525, USA. Fax: +1 732 932 8965.
E-mail address: bini@aesop.rutgers.edu (E. Bini).
[1] Present address: Department of Molecular Biology and Biochemistry, Rutgers University, USA.
[†] Deceased.

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the cells have been monitored over time. Based on the data obtained, a preliminary model for the maintenance of copper homeostasis in *Sulfolbus* is proposed.

Materials and methods

Growth conditions. Sulfolobus solfataricus strains 98/2 or P2 (DSM 1617) were cultured at 80 °C in a defined standard medium (SM) as described in [20], the medium was supplemented with 0.2% sucrose as the carbon and energy source. Batch cultures were inoculated to obtain a density corresponding to an OD_{540} of about 0.025, with aliquots withdrawn from mid-log phase cultures. Growth was monitored at a wavelength of 540 nm on a Beckman DU-520 spectrophotometer (Beckman Coulter, USA). All the experiments were carried out on the strain 98/2, unless otherwise stated.

RNA extraction. Total RNA was isolated from *S. solfataricus* cultures in their exponential phase of growth (OD_{540} = 0.3–0.6). Before centrifugation at 3500*g* for 15 min, cells were mixed with two volumes of RNA Protect (Qiagen, USA). RNA was extracted from the cell pellets using the RNAeasy Mini kit (Qiagen, USA) and treated with DNase (Ambion, USA), as recommended by the manufacturer. DNA contamination was excluded by PCR using primers targeting the 16S rRNA gene. The quantity and quality of the RNA obtained was evaluated both spectrophotometrically on a NanoDrop ND-1000 spectrophotometer (NanoDrop, USA) and by agarose gel electrophoresis [21].

Reverse-transcription PCR (RT-PCR) and real-time quantitative RT-PCR (qRT-PCR) analyses. For RT-PCR analysis, total RNA (0.5 μg) was analyzed in 25-μl reactions using the Enhanced Avian HS RT-PCR Kit (Sigma–Aldrich, USA). The amplification products were separated on a 1.2% agarose gel by electrophoresis, and the gel images were acquired using a GelLogic 440 Imaging System (Eastman Kodak, USA). Specific transcripts were quantified by qRT-PCR using the iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad, USA) and the real-time detection system iCycler iQ (Bio-Rad, USA). Reactions, in triplicate, were assembled according to the protocol of the manufacturer, and using 0.2 μg of total RNA in a 25-μl reaction. Primers were designed using the OligoPerfect Designer software (Invitrogen, USA) to have a composition that was suitable for use in both RT-PCR and qRT-PCR (Table 1). Specificity of each pair of primers was confirmed by sequencing. The efficiency of the PCR amplifications was determined from the slopes of the dilution curves of the target RNA. The cycle threshold (C_t) values obtained were used in the "$2^{-\Delta\Delta Ct}$ Method" to calculate the relative changes in gene expression [22]. Expression of the target RNAs of interest was normalized to the level of the Sso0067 transcript, detected using the primers q0067-F and q0067-R (Table 1). Sso0067 encodes a ribosomal protein and its expression is not affected by copper exposure, as determined by microarray analysis (unpublished).

Analysis of copper content. Cell samples were harvested from exponentially growing *S. solfataricus* cultures. Cell pellets were washed with 10 mM EDTA to remove the copper adsorbed to the cell wall, then rinsed with SM without added trace metals (Zn, Cu, Mo, V, and Co). Before centrifugation at 3500 g for 15 min, aliquots were removed from each sample for determination of protein concentration using the BCA Protein Assay (Pierce, USA). Cell pellets were resuspended in 50% nitric acid, and digested for 16 h at 25 °C. The total copper content was analyzed using a Vista Pro radial view Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, Varian Inc., USA). Controls included non-inoculated SM medium and untreated cultures.

Table 1
Oligonucleotides used in this work.

ID	Sequence (5′–3′)	Target
q2651-F	GAATAGTTGGGATGCATTGT	*copA*
q2651-R	ACTACCCCCTTAACGTTTTC	
q2652-F	TTTATTGCCTTCGCCATTTC	*copR*
q2652-R	GTTGCGTGCAAATTTTTCCT	
2652-F	TGCAATTCTTGCTTGTCTGG	*cop* operon (paired with q2651-R)
q10823-F	ATGATAATCGATCCGGTTTG	*copT*
q10823-R	ATTCCTTAAATACTCTTCCGGA	
q0067-F	TACCAATTGTCGCTTTTGCT	Reference transcript
q0067-R	CAAATCACCATCTGGAGGAA	

Results and discussion

Physiological response to copper

To establish the optimal concentration of copper to be used in this study, cells were exposed to $CuCl_2$ at concentrations ranging from 0 mM to 2.5 mM. The MIC, defined as the lowest concentration that completely inhibits cell growth immediately after exposure, was determined to be 1.5 mM (Fig. 1A). In response to copper concentrations that were equal or greater than 1.5-mM $CuCl_2$, a lag phase of variable duration was observed. The duration of the lag phase was directly proportional to the metal concentration, and growth resumed thereafter, indicating a slow adaptation to levels of copper that were above the MIC. To rule out the possibility that this effect was due to the appearance of copper-resistant mutants, cells adapted to 2-mM copper-containing medium were subcultured in fresh medium in the absence of the metal. After 4 cycles of cell divisions, the cells were re-inoculated into fresh medium containing 2 mM copper. A lag phase was observed again, indi-

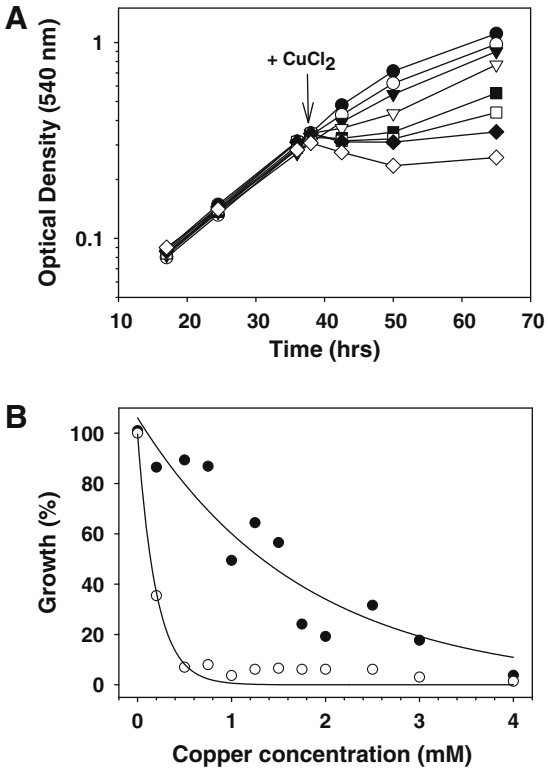

Fig. 1. Effect of $CuCl_2$ on growth of *S. solfataricus*. (A) An exponentially growing culture of strain 98/2 was used as inoculum for 8 subcultures. Copper was added at the time indicated by the arrow, at the following final concentrations: (●), 0 mM; (○), 0.75 mM; (▼), 1 mM; (▽), 1.25 mM; (■), 1.5 mM; (□), 1.75 mM; (♦), 2 mM; (◇), 2.5 mM. (B) Growth of P2 (○) or 98/2 (●). Copper was added at the time of inoculum and cell growth is expressed as percentage of the untreated control. Best fit curves were obtained by nonlinear regression applied to both sets of data.