CopR of *Sulfolobus solfataricus* represents a novel class of archaeal-specific copper-responsive activators of transcription

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In trace amounts, copper is essential for the function of key enzymes in prokaryotes and eukaryotes. Organisms have developed sophisticated mechanisms to control the cytosolic level of the metal, manage its toxicity and survive in copper-rich environments. Here we show that the *Sulfolobus* CopR represents a novel class of copper-responsive regulators, unique to the archaeal domain. Furthermore, by disruption of the ORF Sso2652 (*copR*) of the *Sulfolobus solfataricus* genome, we demonstrate that the gene encodes a transcriptional activator of the copper-transporting ATPase *CopA* gene and co-transcribed *copT*, encoding a putative copper-binding protein. Disruption resulted in a loss of copper tolerance in two *copR*-knockout mutants, while metals such as zinc, cadmium and chromium did not affect their growth. Copper sensitivity in the mutant was linked to insufficient levels of expression of *CopA* and *CopT*. The findings were further supported by time-course inductively coupled plasma optical emission spectrometry measurements, whereby continued accumulation of copper in the *S. solfataricus* mutant was observed. In contrast, copper accumulation in the wild-type stabilized after reaching approximately 6 pg (μg total protein)−1. Complementation of the disrupted mutant with a wild-type copy of the *copR* gene restored the wild-type phenotype with respect to the physiological and transcriptional response to copper. These observations, taken together, lead us to propose that CopR is an activator of *copT* and *copA* transcription, and the member of a novel class of copper-responsive regulators.

INTRODUCTION

Copper is an important trace element which can be found in biological systems in two oxidation states, Cu(I) and Cu(II). Its ability to change oxidation state makes it an essential cofactor in enzymes that support a wide variety of biological processes, including respiration, oxidative stress protection, and metabolism of sugars and aromatic compounds (Zhang & Gladyshev, 2010). Excess copper is highly toxic and its cytosolic level is tightly regulated. Proposed mechanisms for copper toxicity include the formation of reactive oxygen species (Valko et al., 2005), direct binding of copper to lipids, nucleic acids and thiol groups of proteins, and disruption of iron–sulfur clusters of dehydratases (Chillappagari et al., 2010; Macomber & Imlay, 2009). A common strategy to reduce excess intracellular copper is to engage in the sequestration and active efflux of the metal through metal-binding proteins and copper-transporting ATPases, respectively (Rosen, 2002). Expression of the genetic determinants of copper resistance is regulated at the level of transcription by copper-responsive regulators in all three domains of life (Rensing & Grass, 2003; Solioz & Stoyanov, 2003).

In bacteria, copper-responsive regulators can be divided into four main groups, according to their structural features and mechanisms of action. Well-studied copper-responsive regulators belonging to the MerR family include CueR, which mediates copper-induced transcription in *Escherichia coli* (Outten et al., 2000; Stoyanov et al., 2001),
Bacillus subtilis, Cupriavidus metallidurans and Pseudomonas (Gaballa et al., 2003; Julian et al., 2009; Thaden et al., 2010), while HmrR is the CueR homologue in Rhizobium leguminosarum (Reeve et al., 2002). CueR regulators are dimeric, two-domain proteins containing a winged helix–turn–helix (wHTH) DNA-binding domain and a copper-binding effector domain separated by a linker element. Their DNA-binding domains are related to those found in regulators of the MerR superfamily, which bind to their target promoter both in the presence and in the absence of their effector, and act on the topology of the promoter to induce transcription in the presence of copper or repress it in its absence (Reeve et al., 2002; Stoyanov et al., 2001; Thaden et al., 2010). A second type of bacterial copper-responsive regulator includes CopY of Enterococcus hirae and CopY (formerly CopR) of Lactococcus lactis (Cantini et al., 2009; Magnani et al., 2008; Portmann et al., 2004). Both are metal-fist-type repressors belonging to the CopY/TcrY family, and bind to DNA in the form of dimers stabilized by zinc. In the presence of copper, zinc is displaced and the repressor is released from the promoter, allowing transcription (Hasman, 2005; Portmann et al., 2004). Another family of copper-responsive repressors widespread in bacteria, DUF156, is represented by CsoR of Mycobacterium tuberculosis (Liu et al., 2007; Ma et al., 2009), its only characterized member. Finally, bacteria also possess copper-responsive two-component systems, which include the well-characterized CusRS and PcoRS found in E. coli (Brown et al., 1995; Munson et al., 2000; Rouch & Brown, 1997), and PcoRS and CopRS of Pseudomonas syringae (Mills et al., 1993), which are required for the activation of copper-inducible genes.

Although the mechanisms of copper resistance have been thoroughly studied in bacteria (Rensing & Grass, 2003; Soliz & Stoyanov, 2003), this process is still poorly understood in archaea. Investigations of the archaeal response to changes of copper levels are still limited to a few species. Structural studies of individual functional domains of the copper-transporting ATPase CopA in Archaeoglobus fulgidus have provided useful insight into the activities and Cu(I)-exporting functions of the ATPase (González-Guerrero & Argüello, 2008). In Ferroplasma acidarmanus (strain Fer1), copper-dependent expression of the copper-binding protein CopZ and the copper-transporting ATPase CopB have been reported (Baker-Austin et al., 2005), and in Sulfolobus metallicus a novel system for copper detoxification has been described, which is based on sequestration by organic phosphate, possibly followed by active efflux of the metal–phosphate complex (Remonsellez et al., 2006). However, the regulation of copper-responsive expression remains largely uncharacterized in archaea, presumably because in vivo studies of regulated transcription are limited by the availability of genetic systems for micro-organisms belonging to this domain.

The Sulfolobus solfataricus genome contains gene sequences for two metal-transporting ATPases: Sso2651 and Sso2896, encoding CopA and CopB, respectively (Villafane et al., 2009). The two ATPases display high sequence similarity and the typical architecture of both prokaryotic and eukaryotic copper-transporting ATPases (Bini, 2008). The cop locus of S. solfataricus, referred to as copRTA in strain 98/2 (Villafane et al., 2009) and copTMA in strain P2 (Ettema et al., 2006), includes the three ORFs Sso2651, Sso2652 and Sso10823, encoding the CopA ATPase, a copper-responsive regulator and a putative copper-binding protein, respectively. It has previously been shown that the whole operon is co-transcribed at low levels from the copR promoter under all conditions, whereas increased transcription from the copTA promoter occurs in the presence of excess copper (Villafane et al., 2009). In vitro assays show that the regulator binds extensive regions up- and downstream from the putative TATA-box of the copTA promoter, but a conserved consensus has yet to be identified (Ettema et al., 2006). The DNA-binding properties of CopR are consistent with its role as a regulator of transcription; moreover, the fact that the copR transcript is not affected by copper indicates that the function of CopR in response to changes in copper levels is probably accomplished through an allosteric mechanism (Ettema et al., 2006; Villafane et al., 2009).

Here we describe a novel family of copper-responsive regulators that appears unique to the archaeal domain. We also report for the first time that CopR, a member of this family, controls the transcription of copTA by acting as a positive regulator of transcription in S. solfataricus cells challenged with copper. To unravel the role of CopR, mutants carrying a disruption of the copR gene were created. The phenotypes of the mutants, parent strain and complemented mutants were examined with respect to their physiological and transcriptional response to copper. These results led us to formulate a model for the regulation of copper-responsive genes in Sulfolobus.

**METHODS**

**Growth conditions.** S. solfataricus strain 98/2, the ΔlacS mutant PBL2025 (Haseltine et al., 1999), the ΔlacS, ΔΔluxA mutant PBL2069 (Maezato et al., 2011), and derivative strains PBL2050 and PBL2090 constructed as described in this work, were cultured at 80 °C in a defined standard medium (SM), as previously reported (Villafane et al., 2009; Table 1), supplemented with 0.2 % sucrose, unless otherwise noted. Growth was monitored at a wavelength of 540 nm on a Beckman DU-520 spectrophotometer (Beckman Coulter).

**General molecular biology methods.** PCRs were set up using 250 ng genomic DNA in 25 μl reactions with the JumpStart AccuTaq LA DNA polymerase (Sigma-Aldrich), and following the protocol recommended by the manufacturer. Amplification products were separated on a 0.8 % agarose gel by electrophoresis, and the gel images were acquired using a GelLogic 440 imaging system (Eastman Kodak). Where necessary, PCR products were purified using a PCR Clean-Up system (Promega) or a Qiagen II Gel Extraction kit (Qiagen). Cloning, selection and plasmid isolation were performed according to standard procedures (Sambrook et al., 1989). Sequencing was performed by GeneWiz.

**Generation of knockout mutants.** Overlap extension PCR was used to combine three PCR fragments to create the disruption construct.
S. solfataricus strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
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<tr>
<td>98/2</td>
<td>Wild-type</td>
<td>Haseltine <em>et al.</em> (1999)</td>
</tr>
<tr>
<td>PBL2025</td>
<td>AlacS derivative of 98/2</td>
<td>This work</td>
</tr>
<tr>
<td>PBL2050</td>
<td>PBL2025 (copR::lacS); Cu&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Maezato <em>et al.</em> (2011)</td>
</tr>
<tr>
<td>PBL2069</td>
<td>AlacS AmalA derivative of PBL2025</td>
<td>This work</td>
</tr>
<tr>
<td>PBL2090</td>
<td>PBL2069 (copR::lacS); Cu&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>PBL2090/plcopR</td>
<td>pBL2090 complemented with plcopR</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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</tr>
<tr>
<td>pUCdis</td>
<td>pUC derivative containing the disruption construct inserted into BamHI sites; Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pJlacS</td>
<td>Sulfolobus–E. coli shuttle vector; Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Berkner <em>et al.</em> (2007)</td>
</tr>
<tr>
<td>pBN1080</td>
<td>pJlacS derivative constructed by replacing lacS with malA; Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Maezato <em>et al.</em> (2011)</td>
</tr>
<tr>
<td>plcopR</td>
<td>pBN1080 derivative containing copR, PCR-amplified from 98/2 with primers copRcomp-F/R, inserted into KpnI sites; Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
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</table>

Complementation of PBL2090 with a wild-type copy of copR. PBL2090 was transformed with plcopR, a derivative of pBN1080, obtained by replacing the lacS gene of pJlacS (Berkner *et al.*, 2007) with malA (Maezato *et al.*, 2011). plcopR carries a wild-type copy of copR, including its own promoter, inserted at the unique KpnI site of pBN1080 (Supplementary Fig. S2a). After selection on malose, the phenotype was validated by PCR, plasmid extraction and plasmid analysis (Supplementary Fig. S2b).

Sequence analysis. Similarity searches were conducted using BLAST at the National Center for Biotechnology Information (NCBI). Sequences were aligned with CLUSTAL W, and the alignment was manually edited using BioEdit. Programs of the PHYLIP package were used for the analysis. The alignment was resampled 100 times with SEQBOOT. A matrix of distances derived using the Jones–Taylor–Thorton method implemented in PROTDIST was used for the construction of a tree with the neighbor-joining (NJ) program (Felsenstein, 2004).

RNA isolation and analysis. Total RNA was isolated from S. solfataricus cultures in their exponential phase of growth (OD<sub>600</sub> 0.3–0.6), as described previously (Villafane *et al.*, 2009). The RNA was evaluated both spectrophotometrically using a NanoDrop ND-1000 spectrophotometer and by agarose gel electrophoresis (Sambrook *et al.*, 1989). Gel images were acquired using a GelLogic 440 imaging system (Eastman Kodak). Specific transcripts were quantified by quantitative (q)RT-PCR using the iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad) and the real-time detection system iCycler iQ (Bio-Rad), using primer pairs q2651-F/R and q0067-F/R, which detect copA and the reference transcript, respectively (Villafane *et al.*, 2009). The 2<sup>ΔΔCt</sup> method was used to calculate relative changes in gene expression (Livak & Schmittgen, 2001).

RESULTS

The Sulfolobus CopR belongs to a novel class of Cu-responsive regulators

The cop operon of S. solfataricus encodes three genes transcribed from the same strand, named copR, copT and copA (Supplementary Fig. S1a). The copA gene encodes a Cu(I)-transporting ATPase (Bini, 2008) and copT encodes a hypothetical metal-binding protein, probably a copper chaperone. CopR is characterized by the presence of two functional domains: a DNA-binding domain encompassing the N-terminal two-thirds of the protein, and a C-terminal metal-binding domain. The DNA-binding portion of CopR is a wHTH domain, the most frequent HTH domain found in archaea (Aravind & Koonin, 1999). Proteins possessing such a motif are globally grouped within the HTH_GntR superfamily and bind DNA as dimers, and the interaction with an effector molecule most likely affects their affinity for the DNA. The metal-binding effector domain of CopR is constituted by the TRASH domain (transport, resistance and sensing of heavy metals), a
conserved motif predicted to bind metals (Ettema et al., 2003). While the wHTH domain is shared by transcriptional regulators involved in the control of the most diverse metabolic activities, and the TRASH domain is found in proteins with various functions (Ettema et al., 2003), we observed that the particular combination of wHTH and TRASH domains appears to be present only in copper-responsive regulators. Furthermore, this points to an archaeal-specific combination of motifs, as shown by the results of BLASTP searches of the non-redundant database of proteins at NCBI, using the S. solfataricus CopR as query sequence. At the time of writing (May 2011), hits with ≥ 80% sequence coverage included only archaeal sequences. Specifically, 25 hits, with expected value (E), 6e-06 and identity ranging from 25 to 93% (median 32%), were represented by Crenarchaeota or the Euryarchaeota classes Thermoprotei, Thermoplasmata and Archaeoglobi, and inspection of the genomic context surrounding these sequences revealed that all were associated with a metal-transporting ATPase. Twelve additional putative copper-responsive regulator sequences, displaying both domains and association with copper transporters, were retrieved using the Haloarcula marismortui sequence YP_135231 (Euryarchaeota, Halobacteria). In contrast to other Archaea, Halobacteria also possess CopR-like regulators associated with sequences not directly related to copper resistance. Interestingly, if the search was repeated excluding archaeal sequences from the target database, then the most similar sequences retrieved belonged to proteins that shared only the wHTH domain with CopR, and which were unrelated to copper metabolism. Finally, a complementary experiment was performed by restricting the search to the archaeal sequences in the database, and using as query sequences those of characterized bacterial copper-responsive regulators belonging to the CopY/TcrY family, to the MerR family or to DUF156. Hits to the CopY/TcrY family were not significant. Seven MerR-like sequences were retrieved with an E value < 2e-04, but no sequences related to copper resistance were identified when we examined the genome context surrounding the MerR family hits. Similarity to MerR-like sequences was restricted to the DNA-binding domain. With DUF156 as query sequence, four hits were obtained with an E value lower than 2e-04, of which only two, corresponding to Methanobrevibacter ruminantium and Methanobrevibacter smithii, were associated with a cation-transporting ATPase. As both micro-organisms are gut archaea, they may have acquired the copper-responsive genes by lateral transfer, given their shared niche with bacteria. To examine the evolutionary relationships among the different families of regulators, an NJ distance analysis was carried out using the sequences of characterized and putative copper-responsive regulators. The latter were selected for NJ analysis if they satisfied two requirements: amino acid similarity ≥ 50% to any of the characterized regulators over ≥ 80% of their sequence, and a coding sequence near a metal-transporting ATPase gene. The

Table 2. Oligonucleotides used in this work

Underlined nucleotides represent BamHI restriction sites.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’–3’)</th>
<th>Purpose</th>
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<tbody>
<tr>
<td>Bam-F</td>
<td>TTTGATCCCTAAAAGCTGATACATAG</td>
<td>Knockout construct</td>
</tr>
<tr>
<td>B</td>
<td>AATAATATGAAAATAAACGAGCTTTTTGATAGTTCAAGTGAC</td>
<td>Knockout construct</td>
</tr>
<tr>
<td>C</td>
<td>GTACGTGAACATCAAAGGCCTTGTTATGTTTCAAATAATTT</td>
<td>Knockout construct</td>
</tr>
<tr>
<td>D</td>
<td>GCCGATCCTCCTGATACATAG</td>
<td>Knockout construct</td>
</tr>
<tr>
<td>E</td>
<td>CGTCACCTTGGACAAGATTCA</td>
<td>Knockout construct</td>
</tr>
<tr>
<td>Bam-R</td>
<td>GCCGATCCTCCTGATACATAG</td>
<td>Knockout construct</td>
</tr>
<tr>
<td>q2651-F</td>
<td>GAATATGAGGAAGATCAATTGTCATTGTTAAT</td>
<td>qRT-PCR (copA)</td>
</tr>
<tr>
<td>q2651-R</td>
<td>ACTACCCCTTTAAACGTTTCC</td>
<td>qRT-PCR (copA)</td>
</tr>
<tr>
<td>q0067-F</td>
<td>TACCAAATGAGTTTGTGCTTTTGTTC</td>
<td>qRT-PCR (reference gene)</td>
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<tr>
<td>q0067-R</td>
<td>CAAATACACACCGGAGGG</td>
<td>qRT-PCR (reference gene)</td>
</tr>
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<td>M13+pUC</td>
<td>GCCAGGGTTTTTCCAGTGACGAC</td>
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<td>M13Rev-pUC</td>
<td>AGCCGATAACAATTTCACACAGGA</td>
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</tr>
<tr>
<td>LacS-F</td>
<td>TTTTATGTTTCAAATAATTTTAT</td>
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</tr>
<tr>
<td>LacS-R</td>
<td>CTTTCAATTAAGCTAAGTTAAT</td>
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</tr>
<tr>
<td>copRcomp-F</td>
<td>TAGAGGATCCAGTGGATGGAATAG</td>
<td>copR cloning into pBN1080</td>
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<tr>
<td>copRcomp-R</td>
<td>AATTAGGATCCAGTGGATGGAATAG</td>
<td>copR cloning into pBN1080</td>
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<tr>
<td>2652His-F</td>
<td>GACCAATGAGAGAAATGTCAGATTAG</td>
<td>Validation of disruption, detection of pJcopR</td>
</tr>
<tr>
<td>2652His-R</td>
<td>TCTACGTGAGATGTAAGTCGCAAGCTTATTTCG</td>
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<td>Cop5-R</td>
<td>CGTCACCTTGGACAAGATTCA</td>
<td>Validation of disruption</td>
</tr>
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</table>
sequences of single-component regulators clustered in four well-defined clades, further supporting the existence of four distinct classes of regulators (Fig. 1), and the halobacterial sequences formed a separate cluster within the archaeal clade (Fig. 1).

PBL2050 is sensitive to copper but not to other metals tested

To elucidate the role of CopR in the physiological response to copper, strains PBL2025 (wild-type) and PBL2050

![NJ distance tree constructed using known and putative copper-responsive transcriptional regulators. Characterized proteins are underlined. All other sequences used in the tree were found adjacent to cation-transporting ATPase genes. Other than PcoR of *E. coli*, used as outgroup, regulators belonging to two-component systems were not included in the analysis, because of their lack of a sensor domain. Accession numbers are indicated in parentheses. Bootstrap values, based on 100 repetitions, of >50 are shown. Bar, 10% estimated divergence.](image)
(CopR mutant) were exposed to excess CuCl₂. To prevent general stress responses, other than copper-related stress, a sub-lethal concentration of 0.75 mM CuCl₂, which had been previously defined (Villafane et al., 2009), was used. The addition of copper had no effect on growth of strain PBL2025, which behaved as its parent strain 98/2. By contrast, PBL2050 growth was inhibited by the addition of copper, indicating that copR is required for copper resistance (Fig. 2a). A range of copper concentrations, down to 0.1 mM, were tested on this mutant, but even the lowest one inhibited the growth of PBL2050 (data not shown). Given that cadmium (Ettema et al., 2006) and zinc (data not shown) affect the transcription of copA, the growth of mutant PBL2050 was monitored during exposure to these metals to determine whether CopR had any role in their detoxification. The effect of chromium was also tested. However, none of these metals affected the growth of the mutant (Fig. 2b). Because copper has been proposed to cause oxidative stress (Tapiero et al., 2003), we wanted to examine whether the knockout of genes involved in copper detoxification might also have an effect in the response to this type of stress. However, the disruption of copR did not render the mutant strain more sensitive to hydrogen peroxide, as shown by the normal growth of mutant cells that were treated with 0.2 mM H₂O₂ (Fig. 2b). Metals and hydrogen peroxide were used at sub-lethal concentrations, as previously tested on wild-type strains. Our findings in Sulfolobus are thus consistent with those of the Imlay group, showing that no oxidative damage derives from intracellular copper in E. coli (Macomber et al., 2007).

Transcription of copA is not induced in PBL2050

To determine whether CopR has a role in the regulation of copper-responsive genes, expression of copA was measured using qRT-PCR on RNA isolated from cell cultures exposed to copper. The copA gene was expressed at basal levels in untreated cells of both strain PBL2025 and strain PBL2050. However, after copper stress, the level of copA transcript increased approximately 30-fold only in PBL2025 but not in PBL2050, indicating that CopR is involved in the regulation of copA expression, and more specifically, that it is required for induction of copA transcription. This observation is in contrast to the in vitro binding results obtained by Ettema et al. (2006), which suggest a role as repressor for CopR. Because copA is co-transcribed with copT (copM in strain P2) (Ettema et al., 2006; Villafane et al., 2009; Wurtzel et al., 2010), we presume that the regulation of the copper-binding protein CopT is also lost.

PBL2050 accumulates copper above the levels measured in the wild-type

As CopA is a putative copper-transporting ATPase, we speculated that the severe copper sensitivity of the disruption mutants might be due to their inability to efflux the excess metal. Therefore, the total copper associated with the mutant and parent cells was analysed in a time-course experiment by ICP-OES. In the parent strain PBL2025, treated with 0.75 mM copper (or 47.6 × 10⁻³ µg l⁻¹), the level of metal was observed to increase slowly and be maintained at a low level for the period of monitoring (Fig. 3). When the copR disruption mutant PBL2050 was treated with copper, it accumulated significantly more copper than PBL2025 during the 3 h of monitoring (Fig. 3). As previously observed in strain 98/2 (Villafane et al., 2009), the level of copper in PBL2025 increased only slightly and was maintained below 10 pg (µg protein)⁻¹, whereas it continued to accumulate until cell death occurred in the mutant, reaching a concentration of 88.6 pg (µg protein)⁻¹ (Fig. 3).

Fig. 2. Effect of CuCl₂ on growth of parent and knockout mutant strains. (a) Growth curves of PBL2025 and PBL2050 cultures. Copper was added to cell cultures in exponential phase. (b) Growth of the disrupted mutant PBL2050 in the presence of different metal ions or hydrogen peroxide. Cells were treated at the time indicated by the arrow.
The copper-resistant phenotype is restored and the $\text{copA}$ transcript is upregulated in PBL2090/pJcopR

Given that $\text{copR}$ is located upstream of the genes under its control, and all the $\text{cop}$ genes are transcribed in the same direction (Supplementary Fig. S1a), we wanted to verify whether the lack of $\text{copA}$ induction and the increased copper sensitivity of the mutant depended on a polar effect of the disruption of $\text{copR}$ with $\text{lacS}$. We initially attempted to complement PBL2050 with a functional copy of $\text{copR}$. As strain PBL2050 already possesses a copy of the $\text{lacS}$ gene in its disrupted $\text{copR}$ gene, selection of transformants relied on copper resistance, but it was ineffective. Therefore, *S. solfataricus* PBL2069, a $\Delta\text{lacS}$, $\Delta\text{malA}$ mutant (Maertz et al., 2011), was used for generating the $\text{copR}$-knockout PBL2090, a strain with the same genetic background and copper-sensitive phenotype as PBL2050, but with a deletion of the $\alpha$-glucosidase gene ($\text{malA}$) necessary for growth on maltose (see Methods). Strain PBL2090/pJcopR was examined to test whether the wild-type phenotype was restored with respect to copper resistance and ability to regulate $\text{copA}$ expression. PBL2090 and PBL2090/pJcopR cells were exposed to 0.75 mM CuCl$_2$ (Fig. 4). As expected, PBL2090 stopped growing after addition of copper, due to disruption of the $\text{copR}$ gene. The same effect was observed in PBL2050, which has the same genetic background. The addition of copper had no effect on growth of PBL2090/pJcopR on sucrose (Fig. 4), demonstrating that the mutant phenotype is not caused by a polar effect, and that $\text{copR}$ is indeed required for copper resistance in *S. solfataricus*. The physiological response to copper of PBL2090/pJcopR was also tested with maltose as the sole source of carbon and energy. This was not affected by copper stress either. To further show that $\text{copR}$ is responsible for upregulation of the $\text{copA}$ transcript, qRT-PCR was performed using total RNA from PBL2090/pJcopR cell cultures exposed to copper during exponential phase (Fig. 4, inset). The $\text{copA}$ gene is expressed at basal levels in untreated cultures of PBL2090/pJcopR. After treatment with copper for 10 min, the level of $\text{copA}$ transcript increased approximately ninefold. This is consistent with the growth behaviour of PBL2090/pJcopR cells, and the levels of expression are also consistent with those observed with wild-type *S. solfataricus* 98/2 cells in response to copper exposure after a similar exposure time. It was concluded that pJcopR complemented the mutant defect of PBL2090, and that CopR is a copper-responsive activator of $\text{copTA}$ transcription.

**DISCUSSION**

It is well established that the majority of archaeal regulators belong to bacterial families (Aravind & Koonin, 1999), which has raised the long-standing question of how such regulators interact with the eukaryotic-like transcriptional system that characterizes archaea (Geiduschek & Ouhammouch, 2005). Copper-responsive regulation seems to have adapted to the archaeal transcription machinery by assembling components shared with bacteria and eukaryotes to create an effective archaeal-specific transcription factor. *S. solfataricus* CopR possesses a wHTH DNA-binding domain and a copper-binding domain that has been previously described on the basis of sequence homology (Ettema et al., 2003). Although these domains can be individually found in proteins with diverse functions in bacteria and eukaryotes, our comparative
sequence analysis shows the co-occurrence of wHTH and TRASH domains to be restricted to archaeal microorganisms. NJ analysis applied to characterized and putative copper-responsive transcriptional regulators supports a division of these sequences into five distinct groups (Fig. 1). Clustering of the sequences appears to reflect the presence of different functional domains (Fig. 1) and mechanisms of action, in addition to their phylogenetic origin, as exemplified by the two *B. subtilis* regulators falling within the expected groups. The copper-responsive regulator of *Methanobrevibacter ruminantium* is the only archaeal regulator clustering with bacteria, and this could be explained by lateral gene transfer from a bacterial species sharing the rumen environment. Representatives of the four families of proteins identified in bacteria were not found among archaeal sequences and genomes, and conversely we were unable to retrieve any archaeal-like CopR homologues within bacteria. Therefore, we propose that the copper response in archaea is mediated by a novel class of regulators, of which the *Sulfolobus* CopR is the first characterized member.

We demonstrated that CopR is responsible for the transcription of *copTA* in *Sulfolobus*, and determined that it acts as a positive regulator of transcription, through transcriptional analysis of the knockout mutant PBL2050 and physiology analysis of mutant PBL2090, both carrying a disruption of the *copR* gene. In PBL2050, expression of the copper-transporting ATPase gene remains low. This observation is consistent with the copper sensitivity of both mutant strains and suggests that *copR* is an activator of transcription, as its disruption prevents the accumulation of the *copA* transcript. A minimal level of *copA* transcription was still detectable in the mutant. We exclude the possibility that the transcript originates from the disruption cassette, because no promoters are present after the lacS terminator of transcription (Supplementary Fig. S1a). This background expression is extremely low and it becomes evident only in the mutant strain, while in the wild-type it is indistinguishable from the constitutive co-transcription of *copRTA* in the absence of copper (Villafane *et al.*, 2009). Different hypotheses can account for this observation: it may depend on the structure of the *copTA* promoter (a ‘leaky’ promoter that allows minimal transcription in the absence of an activator), or it may indicate the existence of additional factors with a role in *copTA* expression. An intriguing possibility is that CopR might have a dual function, similar to the MerR-like regulator of *E. coli*, as repressor/activator. This would also be supported by earlier studies of the *S. solfataricus* P2 *cop* operon by DNA-protection assays showing that the regulator binds to the *copTA* promoter (copMA in strain P2) regardless of the presence of copper (Ettema *et al.*, 2006), a behaviour reminiscent of the MerR-like mechanism of action.

It is not known whether CopR affects the regulation of genes other than *copTA*, but its disruption makes the cells specifically sensitive to copper, as demonstrated by the fact that growth of the mutant is not inhibited by other metals or H2O2 (Fig. 2b). Growth of the mutant is prevented by levels of copper as low as 0.1 mM, a concentration much lower than the MIC assessed for the wild-type (1 mM), consistent with the observation that copper continues to accumulate in mutant cells, as shown by ICP-OES measurements of copper associated with cells exposed to sub-lethal concentrations of the metal (Fig. 3). Such accumulation can be explained by a failure of a mechanism of detoxification. The wild-type displays a slight accumulation of copper that probably derives from the sequestration of excess intracellular copper by the copper-binding CopT, and possibly other factors acting as copper sinks and yet to be identified. However, the copper associated with the wild-type cell quickly reaches 6 pg (μg protein)^-1^, a value presumably representing the highest tolerable level that can be sustained for a prolonged time.
Previous results were integrated with the data reported in this work to build the tentative model shown in Fig. 5. It is not clear how copper enters the cell. As a specific system for copper uptake has not been identified in the Sulfolobus genome, it is possible that non-specific transporters may be involved. Within the cell, Cu(II) is thought to be spontaneously converted to Cu(I) by the reducing conditions of the cytoplasm. The genes copR, copT and copA are constitutively co-transcribed from the copR promoter (Villafane et al., 2009). In the presence of excess copper, supplementary transcription of copTA is provided by the induction of the copTA promoter, mediated by the transcriptional activator CopR (Fig. 5). A high rate of copTA transcription and CopA and CopT synthesis are necessary to re-establish homeostasis during early copper exposure. As a consequence of the decrease in the intracellular concentration of copper, transcription of copTA declines to reach a low induced level, which is maintained during prolonged exposure to the metal (Villafane et al., 2009). This might be explained by the accumulation of stable CopA and CopT, which would sustain the rate of copper efflux and sequestration in the long term. The copper sensitivity of the copR disruption mutant, consistent with its severe build-up of metal, can be explained by an insufficient amount of the Cu(I) transporter CopA to dispose of the excess copper, and insufficient CopT for its sequestration. Further work is needed to characterize the regulator CopR and the details of its mechanism of action.

At the time of writing (May 2011), 107 complete archaeal genomes were available (versus 1479 bacterial genomes). With a larger representation of archaea within the databases of genome sequences we might be able to confirm the exclusive presence of SsoCopR-like regulators in archaea, and speculate on the existence of this novel class of copper-responsive archaeal regulators in archaea, and provide new insights into the natural history of the specific regulators might be relevant to evolution studies.

Four methods of copper resistance described below are all relevant to the archaea. Interestingly, SsoCopR is conserved in all of these organisms. In Sulfolobus acidocaldarius, multicopy, non-integrative shuttle vectors based on the plasmid pRJ1004. Mol Microbiol 17, 1153–1166.


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