Microbiology (2006), **152**, 1969–1979 DOI 10.1099/mic.0.28724-0

Molecular characterization of a conserved archaeal copper resistance (*cop*) gene cluster and its copper-responsive regulator in *Sulfolobus solfataricus* P2

Thijs J. G. Ettema,† Arie B. Brinkman,‡ Packo P. Lamers, Noor G. Kornet, Willem M. de Vos and John van der Oost

Laboratory of Microbiology, Wageningen University, Hesselink van Suchtelenweg 4, 6703 CT Wageningen, The Netherlands

Correspondence
Thijs J.G. Ettema
T.Ettema@cmbi.ru.nl

Received 28 November 2005
Revised 20 March 2006
Accepted 27 March 2006

Using a comparative genomics approach, a copper resistance gene cluster has been identified in multiple archaeal genomes. The *cop* cluster is predicted to encode a metallochaperone (CopM), a P-type copper-exporting ATPase (CopA) and a novel, archaea-specific transcriptional regulator (CopT) which might control the expression of the *cop* genes. Sequence analysis revealed that CopT has an N-terminal DNA-binding helix–turn–helix domain and a C-terminal TRASH domain; TRASH is a novel domain which has recently been proposed to be uniquely involved in metal-binding in sensors, transporters and trafficking proteins in prokaryotes. The present study describes the molecular characterization of the *cop* gene cluster in the thermoacidophilic crenarchaeon *Sulfolobus solfataricus*. The polycistronic *copMA* transcript was found to accumulate in response to growth-inhibiting copper concentrations, whereas *copT* transcript abundance appeared to be constitutive. DNA-binding assays revealed that CopT binds to the *copMA* promoter at multiple sites, both upstream and downstream of the predicted TATA-BRE site. Copper was found to specifically modulate the affinity of DNA binding by CopT. This study describes a copper-responsive operon in archaea, a new family of archaeal DNA-binding proteins, and supports the idea that this domain plays a prominent role in the archaeal copper response. A model is proposed for copper-responsive transcriptional regulation of the *copMA* gene cluster.

INTRODUCTION

Metal ions play an essential role as trace elements in many biological systems; current estimates indicate that over half of all proteins are metalloproteins, containing metal ions either as a structural component or as a catalytic co-factor (Degtyarenko, 2000). However, at higher concentrations heavy metal ions form aspecific complexes in the cell, leading to toxic effects. Some heavy metal cations, like Hg^{2+}, Cd^{2+} and Ag^{+}, form strong toxic complexes, which makes them unsuitable for physiological function. Also, essential trace elements like Zn^{2+} or Ni^{2+}, and especially Cu^{2+}, are toxic at elevated concentrations (Nies, 1999). Both eukaryotes and prokaryotes have evolved several mechanisms that prevent the cell from being intoxicated by these metals. These homeostasis mechanisms include (i) sequestering of metals by metallothioneins (Blindauer *et al.*, 2002; Camakaris *et al.*, 1999), (ii) the efflux of metals by P-type cation-transporting ATPases (CTAs) (Lutsenko & Kaplan, 1996), and (iii) trafficking of metal ions by metallochaperones (Harrison *et al.*, 2000; Rosenzweig, 2002).

Metal homeostasis has been well studied in bacteria and eukaryotes. In humans, several diseases have been linked to an impaired metal balance, like the Menkes and Wilson's copper storage diseases (Mercer, 2001). In bacteria, several regulatory mechanisms have been identified that ensure a tight regulation of the expression of genes encoding metal homeostasis components. These mechanisms include two-component regulatory systems and metalloregulators. In general, the latter class of proteins comprise a DNA-binding domain and a metal-sensing domain. Examples of bacterial families of metalloregulators are the SmtB/ArsR transcriptional repressors (Busenlehner *et al.*, 2003), the DtxR/MntR family (Guedon & Helmann, 2003) and the MerR family (Brown *et al.*, 2003). These metalloregulatory proteins

†Present address: Centre for Molecular and Biomolecular Informatics, Nijmegen Centre for Molecular Life Sciences, UMC St Radboud, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands.

‡Present address: Department of Molecular Biology, Nijmegen Centre for Molecular Life Sciences, UMC St Radboud, Geert Grooteplein 30, 6525 GA Nijmegen, The Netherlands.

Abbreviations: EMSA, electrophoretic mobility shift assay; HMA, heavy-metal-associated domain; HTH, helix–turn–helix; TBP, TATA-box-binding protein; TFB, transcription factor B.

have been found to display distinct metal selectivity profiles, generating a metal-specific transcriptional response (Busenlehner *et al.*, 2003; Cavet *et al.*, 2003; Guedon & Helmann, 2003). Despite the fact that several archaeal species are able to thrive in environments that contain extremely high metal concentrations (Edwards *et al.*, 2000), information about heavy metal resistance in these organisms is rather limited (Baker-Austin *et al.*, 2005; Dixit *et al.*, 2004; Mana-Capelli *et al.*, 2003; Schelert *et al.*, 2004).

The basal transcription machinery of archaea resembles that of eukaryotes. However, their transcriptional regulatory mechanisms appear to be different. Although a few homologues of eukaryote-like regulators are encoded by archaeal genomes, many potential bacterial-type transcriptional regulators have been identified (Aravind & Koonin, 1999). Apparently, control of the eukaryotic-like transcription machinery in archaea mainly proceeds via bacterial-like regulators. Several studies have revealed that most of these archaeal transcriptional regulators act as repressors of transcription (see Bell, 2005, and references therein). Remarkably, recent studies have also provided evidence of positive control by these regulators (Brinkman *et al.*, 2002; Ouhammouch *et al.*, 2003). In addition to bacterial-like regulators, archaea appear to contain archaea-specific regulators (Gregor & Pfeifer, 2001; Hochheimer *et al.*, 1999).

Recently, we have identified a conserved archaeal gene cluster that comprises a potential copper resistance (*cop*) gene cluster (Ettema *et al.*, 2003). Besides an archaea-specific transcription regulator (*copT*), the cluster consists of genes encoding a putative metallochaperone (*copM*) and a P-type cation-transporting ATPase (*copA*). Interestingly, the CopT, CopM and CopA proteins encoded by the *cop* gene cluster contain a conserved cysteine signature (TRASH domain), implied to play a prominent role in archaeal metal resistance (Ettema *et al.*, 2003). In the present study we have investigated the *cop* gene cluster of the thermoacidophilic crenarchaeon *Sulfolobus solfataricus* to elucidate its molecular function. The genes encoding CopM and CopA were found to be specifically induced upon exposure to copper and, to a lesser extent, upon exposure to cadmium. Furthermore, recombinantly produced CopT protein was found to bind the promoter region of the *copMA* operon in a copper-dependent manner, indicating that CopT is a novel-type archaeal copper-responsive transcriptional regulator of the *cop* cluster.

METHODS

Multiple alignment of archaeal CopT homologues. All protein sequences were retrieved from the Entrez database (NCBI). A multiple alignment of archaeal CopT protein sequences was constructed using the CLUSTALX program (Thompson *et al.*, 1994), followed by manual adjustment for conserved motifs based on BLAST results. Domain analysis of *S. solfataricus* CopT was performed using SMART (Letunic *et al.*, 2004).

Growth of *S. solfataricus*. *S. solfataricus* P2 (DSM1617) was grown aerobically in baffled 250 ml Erlenmeyer flasks filled with 100 ml chemically defined medium containing (l^{-1}) 3·1 g KH_2PO_4, 2·5 g $(NH_4)_2SO_4$, 0·2 g $MgSO_4.7H_2O$, 0·25 g $CaCl_2.2H_2O$, 1·8 mg $MnCl_2.4H_2O$, 4·5 mg $Na_2B_4O_7.10H_2O$, 0·22 mg $ZnSO_4.7H_2O$, 0·06 mg $CuCl_2$, 0·03 mg $Na_2MoO_4.2H_2O$, 0·03 mg $VOSO_4.2H_2O$ and 0·01 mg $CoCl_2$, supplemented with 0·02 g $FeCl_3$ and Wollin vitamins. The Wollin vitamin stock (100×) contained (l^{-1}) 2 mg D-biotin, 2 mg folic acid, 10 mg pyridoxine-HCl, 10 mg riboflavin, 5 mg thiamine-HCl, 5 mg nicotinic acid, 5 mg DL-Ca-pantothenate, 0·1 mg vitamin B12, 5 mg *p*-aminobenzoic acid and 5 mg lipoic acid. The medium was adjusted at room temperature to pH 3·5 with H_2SO_4. After addition of sucrose as carbon source to a final concentration of 0·4 % (w/v) and subsequent inoculation, the culture was propagated at 80 °C in a rotary shaker at 130 r.p.m. Growth was monitored by measuring the optical density at 600 nm.

For determination of the sensitivity of *S. solfataricus* towards various metals, different concentrations of metal salts ($CuSO_4$, Ag_2SO_4, $CdCl_2$, $ZnSO_4$ and $NiSO_4$), as well as EDTA were added to an exponentially growing *S. solfataricus* culture (100 ml culture, OD_{600} ~ 0·3–0·4). The MICs for these compounds were defined as the concentration at which no further growth was observed for a period of 4 h by measuring the optical density at 600 nm.

RNA isolation from *S. solfataricus* and primer extension analysis. *S. solfataricus* total RNA was isolated from mid-exponential-phase cultures (OD_{600} ~ 0·5), grown as indicated, using the RNeasy kit (Qiagen). A sample (50 ml) of culture was washed in 1 ml medium and resuspended in 100 µl TE (10 mM Tris/HCl, 1 mM EDTA, pH 8·0). After the addition of 5 µl 10 % Triton X-100, the RNA was further purified according to the manufacturer's instructions, except that genomic DNA was sheared through a 0·45 mm needle before the sample was applied to a spin column. Columns were eluted twice with 50 µl water. For the determination of the transcription start sites of *copT* and the *copMA* messenger, primer extension analysis was performed using the following radiolabelled antisense oligonucleotides: BG1131 (5′-GTGCTCCTACTGATATTAAGCC-3′) for *copT* and BG1130 (5′-CATGTTGCACAATGCATCCC-3′) for *copMA*. These primers were end-labelled using T4 kinase (Invitrogen) and radioactive [γ-^{32}P]ATP (Amersham Biosciences), according to the manufacturer's instructions. For determination of *copT* and *copMA* expression levels upon addition of various metal salts (see Fig. 4a and b), the same oligonucleotides were used. As an internal control for RNA levels, an antisense oligonucleotide for the *gad* gene (BG2046, 5′-CAGATATAACTCTTAGTGTGGGTAC-3′) was used. The *gad* gene encodes glycolytic gluconate dehydratase (Ahmed *et al.*, 2005) and its expression is expected to be unaffected by the addition of metal salts.

For the primer extension reaction, 10 µg total RNA and 2·5 ng radiolabelled oligonucleotide were resuspended in 2× AMV-RT buffer (Promega) in a final volume of 25 µl. Samples were heated to 70 °C for 10 min and slowly cooled to room temperature. $MgCl_2$, dNTPs, RNasin and AMV-RT (Promega) were added to concentrations of 5 mM, 0·4 mM, 0·8 units μl^{-1} and 0·4 units μl^{-1}, respectively, in a final volume of 50 µl. The samples were incubated at 42 °C for 30 min, extracted with phenol/chloroform, precipitated with ethanol and resuspended in formamide loading buffer. The primer extension products were analysed on an 8 % denaturing sequencing gel along with a sequence ladder that was generated using the same radiolabelled oligonucleotides. After drying, the gels were exposed to a phosphor screen (Amersham Biosciences) and analysed using a molecular imager (Storm 860; Molecular Dynamics). Primer extension products were quantified using ImageQuant software (Molecular Dynamics).

Recombinant production and purification of CopT and LrpA. The *copT* gene was PCR-amplified from *S. solfataricus* genomic DNA using the primers BG864 (5′-CGCGCCATGGAAAAGTTGACAGATTTAGAGTTTAG-3′) and BG865 (5′- CGCGCGGATCCTAATGTAAGTGCAAGCCATTGTTG -3′), which contain *Nco*I and

*Bam*HI restriction sites, respectively (underlined). The generated PCR fragment was cloned into *Nco*I/*Bam*HI-digested pET24d expression vector (Novagen), resulting in pWUR59. The sequence of the cloned *copT* gene was verified by dideoxy sequencing and was subsequently transformed into the *Escherichia coli* expression strain JM109(DE3)-pRIL (Novagen) to produce CopT protein. *E. coli* cells harbouring pWUR59 were grown at 37 °C in 1 l LB medium to an OD_{600} of 0·5, and CopT expression was induced by the addition of IPTG to a final concentration of 0·4 mM. After overnight incubation at 37 °C, the cells were harvested by centrifugation for 10 min at 5000 ***g***. Routinely, a bacterial cell pellet derived from 500 ml expression culture was resuspended in 20 ml buffer P (20 mM Tris/HCl, pH 7·0, 10 mM DTT) containing one complete mini protease inhibitor cocktail tablet (Roche) and subjected to cell lysis by sonification. After cell lysis, the cleared cell lysate was subjected to heat treatment (30 min at 80 °C) and subsequently centrifuged for 30 min at 16 000 ***g***. Recombinant CopT was then purified to apparent homogeneity by size exclusion chromatography using a Superdex 200 10/300 GL column (Amersham Biosciences), which was pre-equilibrated with buffer S (20 mM Tris/HCl, pH 7·0, 0·1 M NaCl, 10 mM DTT). Fractions containing pure CopT were pooled and flushed in an anaerobic chamber to remove residual oxygen molecules. Recombinant CopT was stored at 4 °C. Typically, a 500 ml expression culture harvested approximately 5 mg electrophoretically pure CopT.

Recombinant LrpA from *Pyrococcus furiosus* was produced and purified as described previously (Brinkman *et al.*, 2000).

Electrophoretic mobility shift assays (EMSA) and DNase I footprinting. DNA probes for EMSA were generated using PCR with the primer pair BG1081 (5′-ACTAGTTGGATGGATATTAGGAATAGC-3′) and BG1082 (5′-TCTCTTAAAATCTCCAGCGCTC-3′) for the *copT* promoter fragment (P_{copT}, see also Fig. 5b) and primer pair BG1079 (5′-TGCACGCAACAATGGCTTGC-3′) and BG1725 (5′-GACAATGAGATGAGCAGAAATAG-3′) for the *copMA* promoter fragment (P_{copMA}, see also see Fig. 5b). Subfragments of the *copMA* promoter fragment were generated using primer pair BG1770 (5′-CAATGGCTTGCACTTACATTA-3′) and BG1771 (5′-CTTCTGTAAATTTGTATCTATATA-3′) for P_{copMA}-I, primer pair BG1772 (5′-GAAAAGGTTCTTTATAATAAAAG-3′) and BG1773 (5′-CTAATTATTGCTTTTATTATAAAG-3′) for P_{copMA}-II, and primer pair BG1774 (5′-GATAATCGATCCGGTTTGTGG-3′) and BG1775 (5′-GATTTTTCCCTTATACATTGTC-3′) for P_{copMA}-III (also see Fig. 7a). PCR products were end-labelled using T4 kinase (Invitrogen) and radioactive [γ-^{32}P]ATP (Amersham Biosciences), and purified from a 6 % polyacrylamide gel. Binding reactions were performed in binding buffer B (50 mM Tris/HCl, pH 8·0, 1 mM DTT, 5 % glycerol (w/v), 5 ng poly(dI.dC).poly(dI.dC) μl^{-1}). CopT and metals, if present, were added to final concentrations as indicated and reactions were incubated at room temperature (P_{copMA}-I, P_{copMA}-II, P_{copMA}-III) or at 50 °C (P_{copT}, P_{copMA}) for 20 min. The protein–DNA complexes thus obtained were separated on a non-denaturing 12 % (P_{copMA}-I, P_{copMA}-II, P_{copMA}-III) or 6 % (P_{copT}, P_{copMA}) polyacrylamide gel, buffered in 1 × TBE buffer. Gels were then dried, exposed to a phosphor screen (Amersham Biosciences) and analysed using a molecular imager (Storm 860; Molecular Dynamics). EMSA using *P. furiosus* LrpA was performed as described previously (Brinkman *et al.*, 2000).

For DNase I footprinting, a P_{copMA} probe was generated using PCR with the oligonucleotides BG1079 and BG1725, by end-labelling one of the two oligonucleotides using T4 kinase (Invitrogen) and radioactive [γ-^{32}P]ATP (Amersham Biosciences). Probes were purified from a 6 % polyacrylamide gel. The binding reactions contained 2·5 ng labelled probe (~50 c.p.s.) and 1 μg CopT, and were performed at 50 °C in a total volume of 50 μl containing 50 mM Tris/HCl, pH 8·0, 25 mM $MgCl_2$, 75 mM KCl and 1 mM DTT. After 20 min, the reaction was cooled to 48 °C and 1 μl of a 1 : 50 dilution (approx. 0·6 units) of RNase-free DNase I (Roche) was added. Incubation was then allowed to continue for 1 min. The reaction was terminated by the addition of buffer T (250 μl 10 mM Tris/HCl, pH 8·0, 10 mM EDTA, 750 mM NaCl, 1 % SDS, 0·04 μg glycogen μl^{-1}) and the samples were purified using phenol/chloroform extraction and ethanol precipitation. After resuspension in formamide loading buffer, the samples were analysed on a 6 % denaturing polyacrylamide gel along with a sequence ladder that was generated using the same radiolabelled oligonucleotides.

RESULTS

Analysis of the *S. solfataricus cop* gene cluster

We have previously reported the discovery of a putative archaeal copper (*cop*) resistance gene cluster (Ettema *et al.*, 2003). This *cop* cluster was found to be conserved in several euryarchaeal and crenarchaeal genomes, amongst which is the genome of the aerobic thermoacidophile *S. solfataricus* (Ettema *et al.*, 2003) (Fig. 1). In this organism, the *cop* cluster consists of tandem-orientated genes encoding a putative metallochaperone (*copM*), a P-type cation-transporting ATPase (*copA*) and an archaea-specific transcription regulator (*copT*) (Fig. 1). The *copM* and *copA* genes in *S. solfataricus* overlap by 32 bp, which strongly suggests that these genes are transcribed as a single messenger. Domain architecture analysis reveals that CopT contains an N-terminal helix–turn–helix (HTH) motif (Fig. 2) that resembles the DNA-binding motifs of prokaryotic transcriptional regulators, such as MarR, IclR and Lrp (Brinkman *et al.*, 2003). The presence of these typical prokaryotic HTH motifs has resulted in the mis-annotation of CopT proteins as being members of the Lrp family of transcriptional regulators. However, CopT proteins lack a C-terminal RAM domain (Ettema *et al.*, 2002), which is the characteristic ligand-binding domain of Lrp-like proteins (Brinkman *et al.*, 2003). Instead, the C-terminal part of CopT contains a putative metal-binding TRASH domain (Ettema *et al.*, 2003), consisting of a conserved cysteine motif that is located within a 30 aa region (Fig. 2).

Expression of *cop* genes in *S. solfataricus*

The *cop* operon of *S. solfataricus* consists of the tandemly orientated *copT* gene and the *copMA* gene pair (Fig. 1). To determine the role of the genes of this cluster, the expression of the *cop* genes was analysed under various conditions. First, the MIC of different metals was determined for *S. solfataricus* (Table 1). The obtained MIC values resembled the values reported by Grogan (1989), except for Cd^{2+} and Zn^{2+}, which were found to be slightly higher (Table 1).

To determine the transcription start sites of the *copT* and *copMA* transcripts, we separated the obtained primer extension products on a larger gel along with sequence reactions that were performed with the same *copT* and *copMA* antisense primers. As shown in Fig. 3(a), *copT* transcription is initiated 1 bp upstream of the predicted start codon of the *copT* gene, whereas transcription of *copMA* is initiated 2 bp upstream of the predicted *copM* start

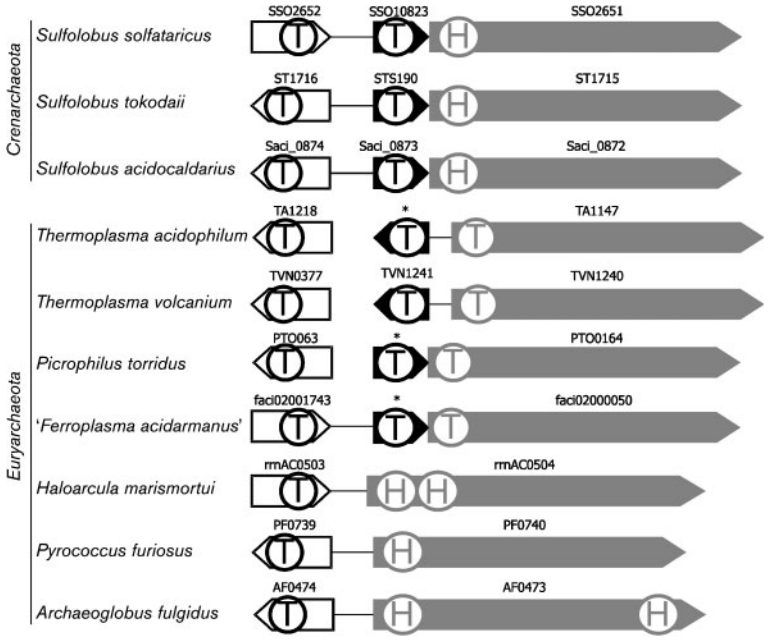

Fig. 1. Conserved gene organization of archaeal *cop* loci. Genes encoding CopT, CopM and CopA are indicated with white, black and grey arrows respectively. Drawn lines indicate a physical linkage between genes. Gene numbers are indicated above the genes. Genes that have been overlooked in genome annotation and are absent from protein databases are indicated with an asterisk. Genes are drawn approximately to scale. Domain abbreviations: T, TRASH domain; H, HMA domain. (Adapted from Ettema *et al*., 2003.)

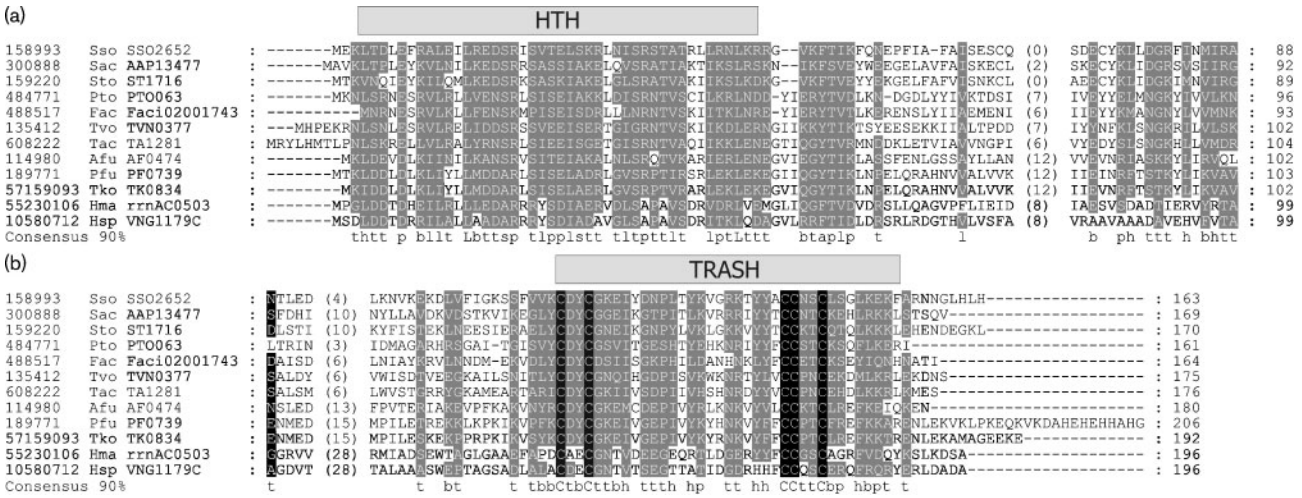

Fig. 2. Sequence analysis of CopT. (a) Multiple alignment of the core of archaeal CopT proteins. The sequences are denoted as in Fig. 1 (except that for *S. acidocaldarius* CopT a locus entry is provided). The positions of the first and the last residue of the aligned region in the corresponding protein are indicated for each sequence. The 90 % consensus sequence shown below the alignments was obtained using the following amino acid classes: aromatic (*a*, FYW), small (*s*, GASC), charged (*p*, STEDKRNQH), turn-forming (*t*, ASTDNVGPENRK), big (*b*, FILMVWYKREQ) and hydrophobic (*h*, ACFILMVWYH), of which the aliphatic subset (*l*, ILVA) residues that show 100 % conservation are indicated in capitals. Residues conserved >90 % are shown in white type on a grey background. Conserved cysteine residues that have been predicted to be involved in metal binding are shown in white type on a black background. The location of a putative HTH motif and the TRASH domain are indicated above the alignment. The numbers within the alignment represent poorly conserved inserts that are not shown. For the CopT sequences of *P. furiosus* (PF0739) and *Halobacterium* sp. (VNG1179C), the transcription starts have been adjusted. Species abbreviations: Afu, *Archaeoglobus fulgidus*; Fac, '*Ferroplasma acidarmanus*'; Hma, *Haloarcula marismortui*; Hsp, *Halobacterium* sp. NRC1; Pto, *Picrophilus torridus*; Pfu, *Pyrococcus furiosus*; Sac, *Sulfolobus acidocaldarius*; Sso, *Sulfolobus solfataricus*; Sto, *Sulfolobus tokodaii*; Tko, *Thermococcus kodakaraensis*; Tac, *Thermoplasma acidophilum*; Tvo, *Thermoplasma volcanium*.

Table 1. MICs of various metal salts for *S. solfataricus*

Salt	MIC (μM)	
	Grogan (1989)	This study
EDTA	–	<1000
$CuSO_4$	5000	5000
Ag_2SO_4	8	8
$CdCl_2$	2000	>4000
$ZnSO_4$	50 000	>75 000
$NiSO_4$	600	600

codon (Fig. 3b). We could not detect bands corresponding to a smaller primer extension product (not shown), suggesting that the *copM* and *copA* genes are transcribed as a polycistronic mRNA, initiated from a single promoter upstream of *copM*. Sequence elements matching *S. solfataricus* consensus TATA boxes and BREs (Bell *et al.*, 1999b) are present upstream of the two mapped transcriptional starts (Fig. 3a, b). The *copMA* genes of '*Ferroplasma acidarmanus*' (referred to as *copZB*) have also been shown to be expressed as a polycistronic messenger (Baker-Austin *et al.*, 2005).

To analyse the transcription regulation of the *cop* operon, different metals were added to an *S. solfataricus* culture that was growing exponentially in defined medium, at a concentration that was equivalent to the determined MIC value. Subsequently, total RNA was isolated and analysed using primer extension analysis using antisense primers for *copT* and *copMA*. As shown in Fig. 4(a), transcription of *copT* is constitutive under the tested conditions, whereas transcription of the *copMA* messenger is induced specifically in the presence of Cu^{2+} and Cd^{2+}. Under non-induced conditions, *copMA* appears to be transcribed at a basal level, which decreases when the metal-chelating agent EDTA was added. In contrast, addition of EDTA had no apparent effect on *copT* expression. It should be noted that addition of 1 mM EDTA immediately abolished growth of *S. solfataricus*. Induction of *copMA* transcription by Cu^{2+} was further studied by isolation of total RNA from an *S. solfataricus* culture at different time intervals. Transcription of *copMA* was significantly induced after 30 min, and the transcript concentration gradually increased during the first 3 h after induction to a level more than 14-fold higher than the basal transcription level (Fig. 4b). To determine whether basal *copMA* transcription is due to the small amount of Cu^{2+} (0·45 μM) present in the defined medium, *copMA* transcription was also determined in defined medium from which Cu^{2+} was omitted. Although no significant decrease in basal transcription was observed (results not shown), we

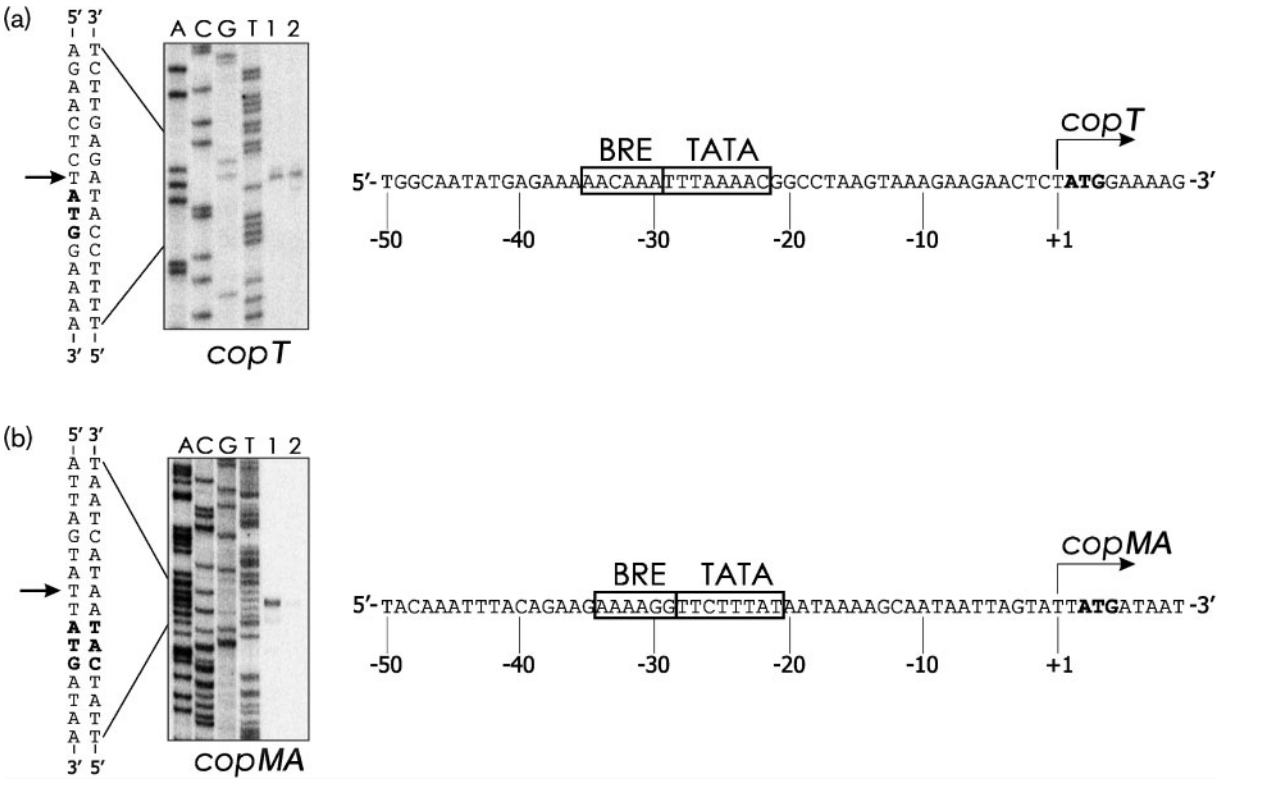

Fig. 3. Mapping of the transcription start sites of *copT* and *copMA* genes. Primer extension products of *copT* (a) and *copMA* (b) were analysed on a sequencing gel along with a sequence ladder. Lanes 1 and 2 contain primer extension products using RNA from $CuSO_4$-induced cells and non-induced cells, respectively. The transcriptional start sites of *copT* and *copMA* are indicated with an arrow (+1), and presumptive BREs and TATA boxes are indicated by rectangles.

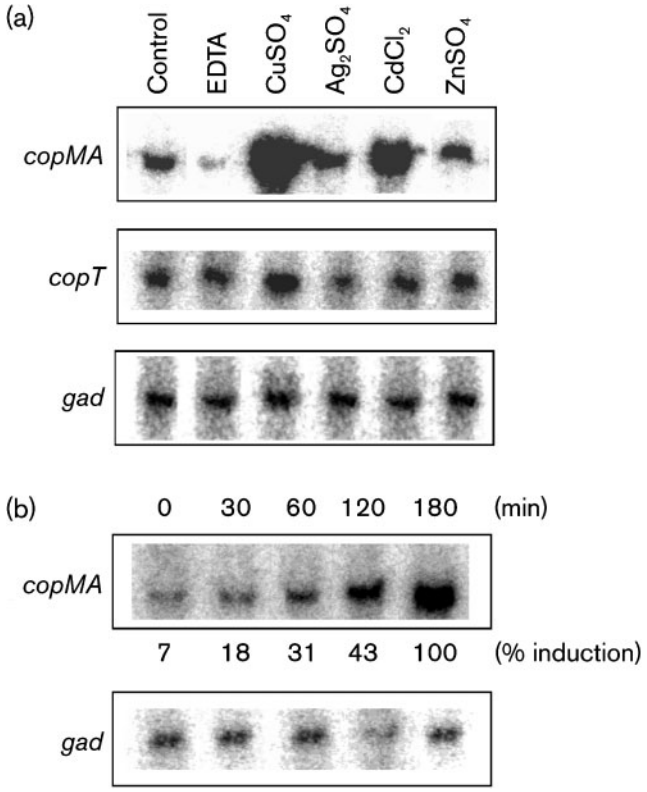

Fig. 4. *copMA* transcript levels accumulate upon exposure to elevated $CuSO_4$ and $CdCl_2$ levels. Effect of addition of different metals on expression levels of *copT* and *copMA* (a). MICs of EDTA and various metals (see Table 1) were added to an exponentially growing *S. solfataricus* culture. EDTA, 1 mM; $CuSO_4$, 5 mM; Ag_2SO_4, 8 μM; $CdCl_2$, 4 mM; $ZnSO_4$, 75 mM. Four hours after addition of EDTA or metals, total RNA was isolated and analysed using primer extension with *copT* and *copMA* antisense primers. (b) Induction of *copMA* transcription by $CuSO_4$ was further studied at different time intervals (0, 30, 60, 120 and 180 min after addition of 5 mM $CuSO_4$). As an internal control, the *gad* (encoding *S. solfataricus* gluconate dehydratase) primer extension product is shown.

cannot rule out the possibility that trace amounts of Cu^{2+} were still present in the demineralized water that was used to prepare the defined medium. These results are in good agreement with the recently published study of copper resistance of another archaeon, '*F. acidarmanus*' (Baker-Austin *et al.*, 2005). In this study, a 15-fold induction of the bicistronic *copMA* transcript (referred to as *copZB*) was reported upon exposure to elevated levels of copper (5 g $CuSO_4$ l^{-1}), whereas the expression of the *copT* gene (referred to as *copY*) was unaffected upon addition of copper (Baker-Austin *et al.*, 2005). In contrast, expression of the '*F. acidarmanus*' *copMA* genes was not induced upon addition of cadmium, like the *copMA* genes of *S. solfataricus*.

We conclude that the genes in the *cop* regulon in *S. solfataricus* are most probably involved in the efflux of copper and cadmium, since these metals specifically induce expression of the *copMA* transcript. As with many transcriptional regulators, *copT* expression appears to be maintained at a constitutive level under the tested conditions.

CopT specifically targets the *copMA* promoter

The *copT* gene was cloned into the pET24d expression vector (Novagen) and functionally overproduced in *E. coli*. Subsequently, CopT was purified to electrophoretic homogeneity as described in Methods (Fig. 5a). To prevent undesired protein aggregation, the purified CopT protein was stored under anoxic conditions in the presence of excess amounts of DTT (10 mM). Subsequently, purified CopT was used in EMSAs to determine whether it binds to the mapped *copT* and *copMA* promoters (P_{copT}, P_{copMA}; Fig. 5b). CopT was unable to bind to the 200 bp P_{copT} fragment under the tested conditions (Fig. 5c). This finding is in agreement with the observation that *copT* expression is constitutive under all tested conditions and suggests that CopT does not autoregulate its own expression. In contrast, transcriptional regulators of well characterized *cop* regulons of *E. coli* (CueR) (Outten *et al.*, 2000; Stoyanov *et al.*, 2001) and *Enterococcus hirae* (CopY) (Strausak & Solioz, 1997) are subjected to autoregulation.

Binding of CopT to a 222 bp DNA fragment containing the *copMA* promoter was observed (Fig. 5e). EMSA experiments revealed that after the first CopT–DNA complex (complex 1) was formed, four additional complexes of distinct electrophoretic mobility were formed with increasing CopT concentration (Fig. 5d). This suggests that multiple CopT-binding sites are present in P_{copMA}.

Cu^{2+} modulates the CopT–DNA interaction

Because transcription analysis showed that both Cu^{2+} and Cd^{2+} induce expression of the *copMA* operon, we investigated the effect of adding metal ions on the formation of CopT–DNA complexes. Addition of Cu^{2+} to the binding reaction was found to reduce the DNA-binding capacity of CopT (Fig. 5d), whereas addition of copper did not affect the binding of an unrelated archaeal transcriptional regulator (LrpA; Brinkman *et al.*, 2000) to its target promoter (P_{lrpA}) (Fig. 5e); this strongly suggests a specific effect of copper on CopT functionality. Although Cd^{2+} induced *copMA* expression (addition of Cd^{2+} only has marginal effects on CopT–DNA complex formation), as reflected by a slight decrease of complex 1, the intensity of complex 5 slightly increased (Fig. 5d).

To study the effect of Cu^{2+} on CopT–DNA complex formation in more detail, EMSA analysis was performed with P_{copMA} and increasing amounts of CopT in either the presence or absence of Cu^{2+} (Fig. 5f). The addition of Cu^{2+} to the binding reaction affected CopT–DNA binding in two ways. First, the overall binding affinity decreased, and second, the formation of complex 1 (Fig. 5f) was completely obliterated. It is proposed that exogenous Cu^{2+} binds

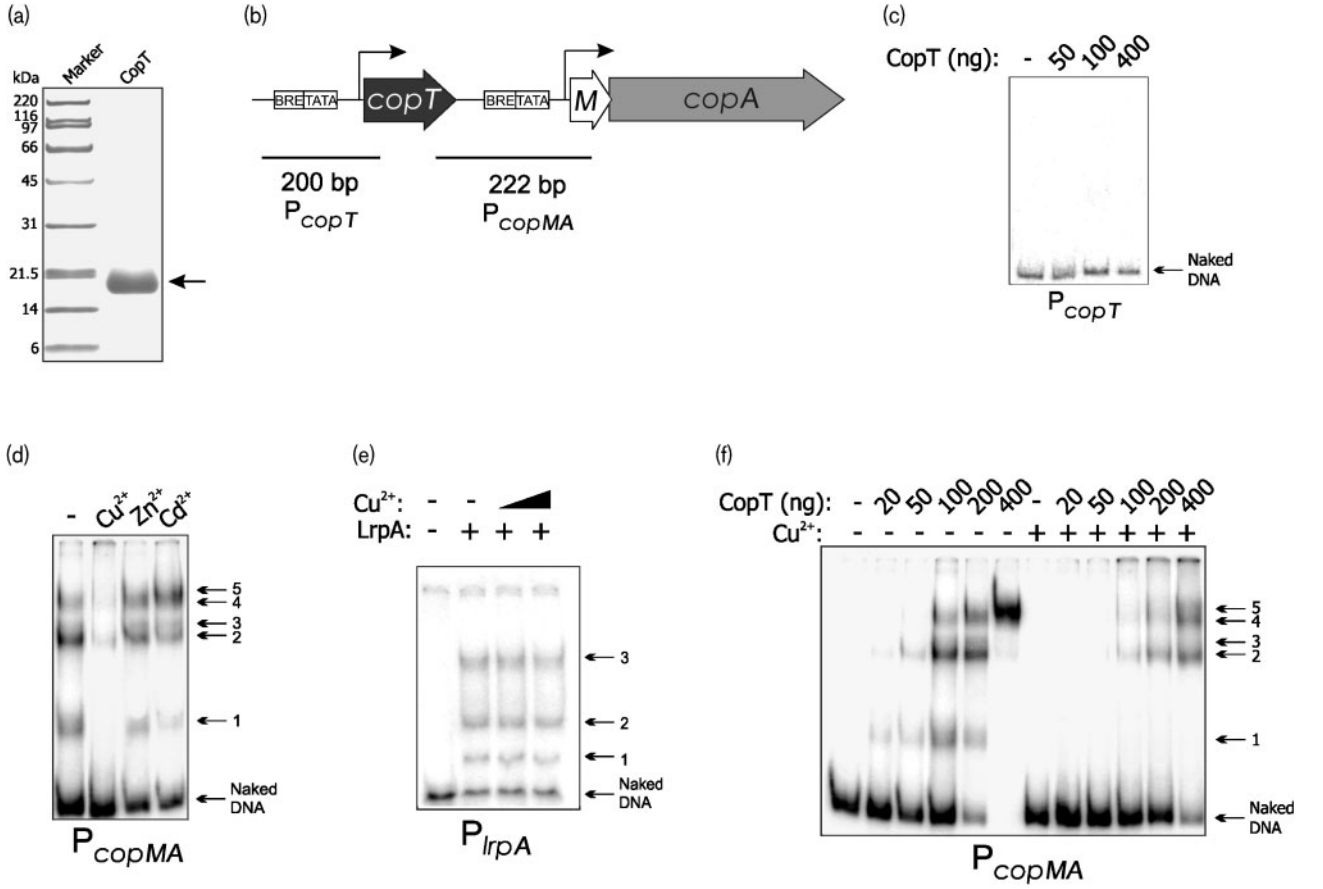

Fig. 5. CopT binds to the *copMA* promoter, but not to its own promoter. (a) SDS-PAGE analysis of purified CopT. The arrow indicates a single band that corresponds to the size of a CopT monomer. (b) Schematic display of the DNA fragments used for EMSAs: P_{copT} and P_{copMA}. Genes are not drawn to scale. (c) EMSA with P_{copT} using increasing amounts of CopT. (d) EMSA with P_{copMA} using increasing amounts of CopT. The five different CopT–DNA complexes are numbered 1–5, respectively. (d) Effects of various metal ions on CopT–DNA binding. Binding reactions were performed as described in Methods and contained 200 ng CopT. Metal salts were added to a final concentration of 100 μM. (e) Control experiment showing that addition of different concentrations of copper ions (0, 10 and 100 μM $CuSO_4$) to an unrelated archaeal transcriptional regulator (LrpA) has no effect on binding to its target promoter, P_{lrpA}. (f) Effect of Cu^{2+} on CopT–DNA binding at different concentrations of CopT. Assays were performed in the presence or absence of 100 μM $CuSO_4$.

to the TRASH domain, to cause an allosteric conformational change in CopT, resulting in an altered DNA-binding mode involving a lower DNA-binding affinity and perhaps different multimerization properties. Consequently, this enables the initiation of transcription of the *copMA* genes. Together with the observed induction of *copMA* transcription upon exposure to excess levels of exogenous copper *in vivo*, it is suggested that CopT is a repressor of *copMA* transcription and that transcription occurs by copper–CopT-mediated derepression.

CopT binds to multiple binding sites at the *copMA* promoter

To determine the locations where CopT interacts with the *copMA* promoter, DNase I footprinting was performed (Fig. 6a). CopT protects P_{copMA} at multiple locations, suggesting the presence of multiple CopT-binding sites (Fig. 6b, c). However, close examination of the protected regions did not reveal the presence of any type of CopT consensus motif. In addition, extensive searches for putative *cop* motifs (including palindromic sequences, direct and indirect repeats and other conserved motifs) in the *cop* gene clusters of other archaeal species (as displayed in Fig. 1) did not result in the identification of any conserved motif (T. J. G. Ettema & J. Van der Oost, unpublished results). The absence of such a motif implies either the existence of a very degenerative *cop* motif or that specific DNA recognition by CopT is accomplished by other, hitherto unknown determinants.

The regions that were protected by CopT in the DNase I footprint assay are located both upstream and downstream of the putative TATA box and BRE sequence, but do not

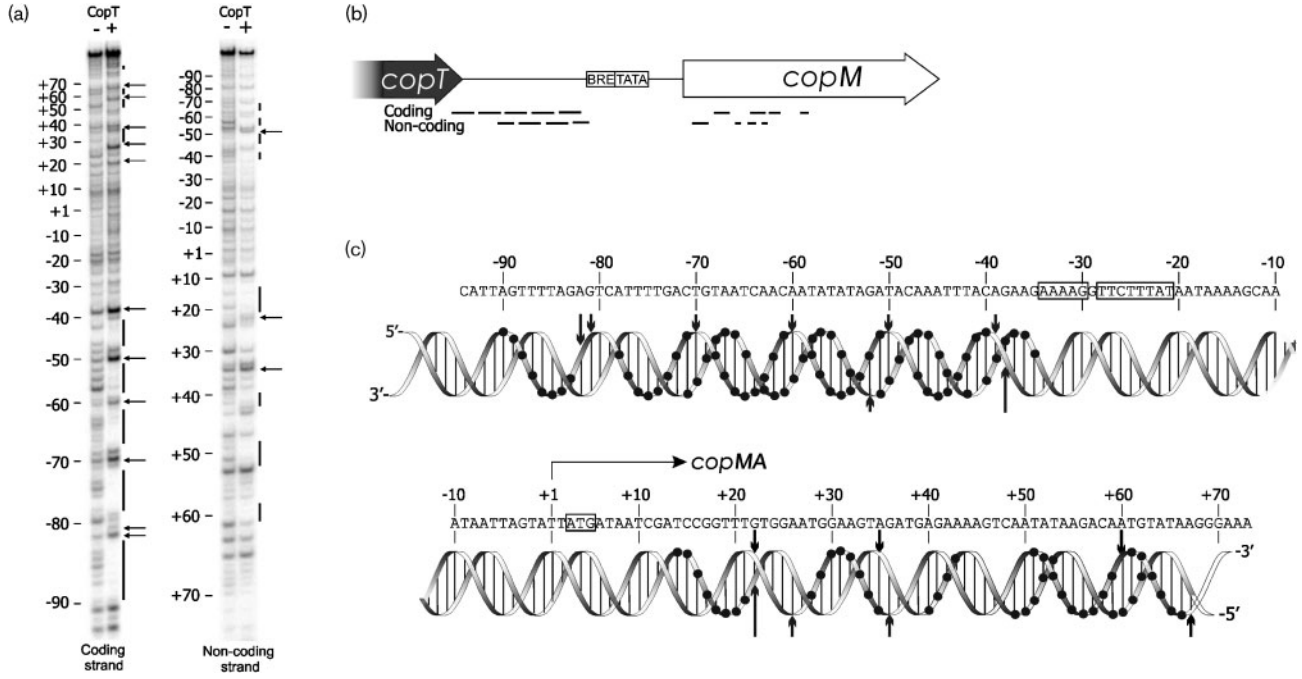

Fig. 6. DNase I footprint analysis of CopT bound to P_{copMA}. (a) A saturating amount of CopT was added in the binding reaction as described in Methods and compared to a reaction lacking CopT. Sequence ladders are shown for the coding and non-coding strand and protected bases are indicated by vertical bars. DNase I hypersensitive sites are indicated by a horizontal arrow. (b) Schematic display of the CopT-induced protected regions of P_{copMA}. The TATA-box and BRE element are shown. (c) Imposition of the DNase I footprinting results on a double-helical representation of the *copMA* promoter region. Filled circles indicate sites that were protected during DNase I degradation and bold arrows indicate DNase I hypersensitive sites. The sequence of the non-template strand is indicated above the helical representation, and boxed sequences indicate the TATA-box, BRE element and start codon (ATG). The sequence positions are relative to the *copMA* transcription start site (+1), which is indicated by an arrow.

overlap with these elements. This suggests that, potentially, the recruitment of basal transcription factors TFB (transcription factor B) and TBP (TATA-box-binding protein) is not prevented by the presence of CopT on P_{copMA}.

To study differential binding affinity of CopT for binding sites up and downstream of the TATA-BRE region, EMSAs were performed with different subfragments of P_{copMA} (Fig. 7a). Indeed, CopT bound only to the fragments upstream and downstream of this TATA-BRE region, but not to a 33 bp P_{copMA} fragment that includes the proposed TATA-BRE site (Fig. 7b, fragment II). This is in agreement with the results from the DNase I footprint assay. Addition of saturating amounts of CopT resulted in two complexes for each promoter fragment (Fig. 7b, fragments I and III). Addition of exogenous copper to the binding reaction appears to have a more severe effect on CopT binding to fragment III than to fragment I. For fragment III (Fig. 7b, right panel), the amount of CopT-bound probe dropped from 88 % (no copper added) to 16 % (500 μM $CuSO_4$ added), whereas only a marginal effect was observed for fragment I, where the amount of CopT-bound probe only dropped from 59 to 45 % upon addition of 500 μM $CuSO_4$ (Fig. 7b, left panel). It is concluded that CopT displays differential binding affinity to the sites on fragments I and III.

DISCUSSION

A copper resistance gene cluster in *S. solfataricus*

The present study describes the molecular characterization of a copper resistance gene cluster and its transcriptional regulator in the crenarchaeon *S. solfataricus*. This *cop* gene cluster is conserved in several archaeal genomes and encodes a putative metallochaperone (*copM*), a P-type cation-transporting ATPase (*copA*) and an archaea-specific transcriptional regulator (*copT*) (Fig. 1). When *S. solfataricus* is challenged by the addition of various metals, we have shown that the polycistronic *copMA* transcript is found to accumulate in response to growth-inhibiting copper and, to some extent, cadmium concentrations, whereas *copT* transcript abundance appeared to be constitutive. Increased levels of *copMA* expression will result in increased levels of CopA and CopM protein. Whereas CopA is most probably involved in copper efflux, as has been shown for its

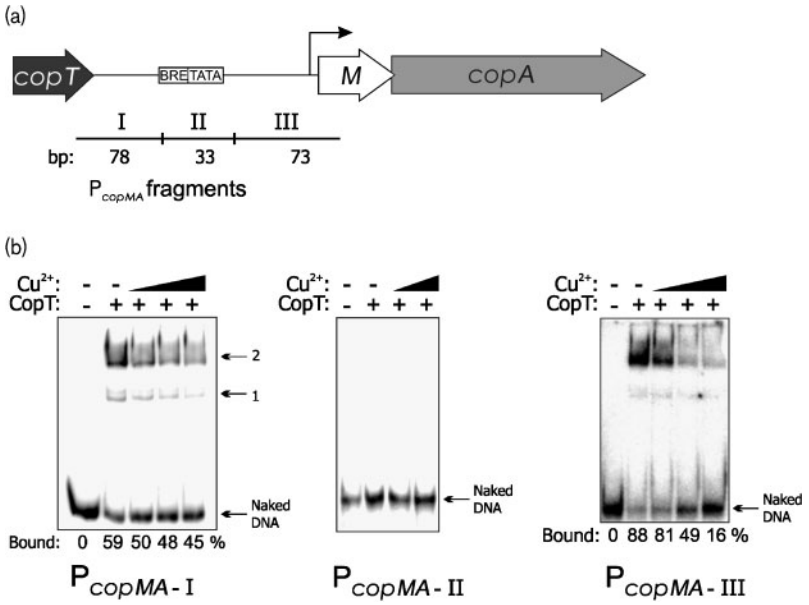

Fig. 7. Binding of CopT to *copMA* promoter fragments up- and downstream of the predicted TATA-box and BRE element is differentially influenced by exogenous Cu^{2+}. (a) Schematic display of the different DNA fragments used for EMSA analysis (P_{copMA}-I, -II and -III). (b) EMSA with CopT, P_{copMA} fragments and $CuSO_4$. Where indicated (+), 400 ng CopT was added to the binding reactions. The different CopT–DNA complexes are indicated. $CuSO_4$ was added to final concentrations of 10, 100 or 500 μM. For fragment P_{copMA}-II, the assay containing 100 μM $CuSO_4$ was not performed.

orthologue CopB in *Archaeoglobus fulgidus* (AF0474) (Mana-Capelli *et al.*, 2003), the role of CopM remains to be established. Similarly to heavy-metal-associated domain (HMA)-type metallochaperones, CopM might play a role (i) in metallotrafficking-like functions (Hung *et al.*, 1998; Multhaup *et al.*, 2001; Tottey *et al.*, 2002), (ii) in regulation of CopA activity (Hamza *et al.*, 1999; Walker *et al.*, 2002), or (iii) in modulation of transcriptional regulation (Cobine *et al.*, 1999, 2002a, b).

The findings described above closely resemble the results recently published by Bond and co-workers, describing a molecular/proteomic study of proteins involved in copper resistance in the extreme acidophilic archaeon '*F. acidarmanus*' Fer1 (Baker-Austin *et al.*, 2005). They reported that the *copM* and *copA* genes (which are referred to in their manuscript as *copZ* and *copB*, respectively) in this organism are transcribed as a polycistronic messenger and are induced in response to high copper levels. Interestingly, the proteomic analysis of copper-induced '*F. acidarmanus*' Fer1 cells revealed an accumulation of proteins involved in protein folding (thermosome subunits, molecular chaperones) and DNA repair. Most probably, this reflects a response to copper-mediated protein misfolding, and/or to free-radical formation (Baker-Austin *et al.*, 2005).

CopT, a copper-responsive transcription regulator of the *cop* gene cluster

The putative transcriptional regulator CopT of *S. solfataricus* was successfully overexpressed in *E. coli* and purified to homogeneity. Subsequent DNA-binding studies revealed that CopT bound to multiple sites on the *copMA* promoter *in vitro*, and this interaction is specifically modulated upon the addition of copper. Coordinated binding of Cu^{2+} ions by the C-terminal TRASH domain of CopT is expected to result in an allosteric change in structural conformation, most likely resulting in a reduced DNA-binding affinity, and perhaps in a different multimeric state. Unfortunately, the creation of site-directed CopT mutant proteins containing single Cys–Ser mutations for each of the invariant cysteine residues was hampered due to the formation of insoluble protein aggregates (T. J. G. Ettema & J. Van der Oost, unpublished results). This reduced the possibility of specifically studying the copper-dependence of DNA-binding by CopT and indicates that, besides playing an important role in selective binding of metal ions, these residues are strongly involved in maintaining the stability of the protein.

The results shown in this study are indicative of a copper-responsive transcriptional mechanism in which expression of the *copMA* messenger is stimulated via transcriptional derepression. This mechanism, which has been demonstrated for several archaeal transcription regulators, is characterized either by prevention of recruitment of the basal transcription factors TBP and TFB, or RNA polymerase, or by acting as a transcriptional roadblock, preventing transcription elongation by the RNA polymerase (Bell, 2005, and references therein).

MDR1 from *A. fulgidus* is the archaeal regulator for which the molecular mechanism of transcription regulation is understood in most detail (Bell *et al.*, 1999a). This transcriptional repressor has been shown to recognize three operator sites on a promoter of a metal ABC transporter gene cluster in the presence of various metals *in vitro* and *in vivo*. Transcriptional repression was found to occur via impaired recruitment of RNA polymerase, while TFB and TBP were allowed to bind to their target sequences, governing a rapid transcriptional response after metal exposure (Bell *et al.*, 1999b). Interestingly, CopT binds to sites both up- and downstream of the predicted TATA-BRE box

(Fig. 6a–c), which is a peculiarity compared to archaeal transcriptional regulators acting as derepressors.

Based on the differential copper-induced binding affinity towards the binding sites upstream (fragment I) and downstream (fragment III) (Fig. 7), it is tempting to speculate that the latter binding sites are mainly involved in transcriptional repression, which is released upon the response to copper. Because CopT binding to the region upstream of the TATA-BRE fragment (fragment I) is less affected by exposure to copper, binding of CopT in this region may be constitutive *in vivo*, where it could enforce a stimulatory affect on transcription, possibly by interacting with basal transcription factors like TFB and TBP. However, the exact mechanism of CopT-mediated transcriptional regulation of *copMA* remains to be elucidated by future experiments.

Finally, it will be a major challenge to study the roles of the individual proteins encoded by the archaeal *cop* clusters, as well as their interactions, to elucidate the mechanism that governs TRASH-domain-mediated copper resistance in the archaea.

ACKNOWLEDGEMENTS

We thank N. J. Robinson (Medical School, Newcastle University) for helpful suggestions and for critically reading the manuscript prior to submission and S. D. Bell (Medical Research Council Cancer Cell Unit, Hutchison MRC Research Centre, Cambridge) for experimental advice.

REFERENCES

Ahmed, H., Ettema, T. J., Tjaden, B., Geerling, A. C., van der Oost, J. & Siebers, B. (2005). The semi-phosphorylative Entner–Doudoroff pathway in hyperthermophilic archaea: a re-evaluation. *Biochem J* **390**, 539–540.

Aravind, L. & Koonin, E. V. (1999). DNA-binding proteins and evolution of transcription regulation in the archaea. *Nucleic Acids Res* **27**, 4658–4670.

Baker-Austin, C., Dopson, M., Wexler, M., Sawers, R. G. & Bond, P. L. (2005). Molecular insight into extreme copper resistance in the extremophilic archaeon '*Ferroplasma acidarmanus*' Fer1. *Microbiology* **151**, 2637–2646.

Bell, S. D. (2005). Archaeal transcriptional regulation – variation on a bacterial theme? *Trends Microbiol* **13**, 262–265.

Bell, S. D., Cairns, S. S., Robson, R. L. & Jackson, S. P. (1999a). Transcriptional regulation of an archaeal operon *in vivo* and *in vitro*. *Mol Cell* **4**, 971–982.

Bell, S. D., Kosa, P. L., Sigler, P. B. & Jackson, S. P. (1999b). Orientation of the transcription preinitiation complex in archaea. *Proc Natl Acad Sci U S A* **96**, 13662–13667.

Blindauer, C. A., Harrison, M. D., Robinson, A. K., Parkinson, J. A., Bowness, P. W., Sadler, P. J. & Robinson, N. J. (2002). Multiple bacteria encode metallothioneins and SmtA-like zinc fingers. *Mol Microbiol* **45**, 1421–1432.

Brinkman, A. B., Dahlke, I., Tuininga, J. E. & 7 other authors (2000). An Lrp-like transcriptional regulator from the archaeon *Pyrococcus furiosus* is negatively autoregulated. *J Biol Chem* **275**, 38160–38169.

Brinkman, A. B., Bell, S. D., Lebbink, R. J., de Vos, W. M. & van der Oost, J. (2002). The *Sulfolobus solfataricus* Lrp-like protein LysM regulates lysine biosynthesis in response to lysine availability. *J Biol Chem* **277**, 29537–29549.

Brinkman, A. B., Ettema, T. J., de Vos, W. M. & van der Oost, J. (2003). The Lrp family of transcriptional regulators. *Mol Microbiol* **48**, 287–294.

Brown, N. L., Stoyanov, J. V., Kidd, S. P. & Hobman, J. L. (2003). The MerR family of transcriptional regulators. *FEMS Microbiol Rev* **27**, 145–163.

Busenlehner, L. S., Pennella, M. A. & Giedroc, D. P. (2003). The SmtB/ArsR family of metalloregulatory transcriptional repressors: structural insights into prokaryotic metal resistance. *FEMS Microbiol Rev* **27**, 131–143.

Camakaris, J., Voskoboinik, I. & Mercer, J. F. (1999). Molecular mechanisms of copper homeostasis. *Biochem Biophys Res Commun* **261**, 225–232.

Cavet, J. S., Borrelly, G. P. & Robinson, N. J. (2003). Zn, Cu and Co in cyanobacteria: selective control of metal availability. *FEMS Microbiol Rev* **27**, 165–181.

Cobine, P., Wickramasinghe, W. A., Harrison, M. D., Weber, T., Solioz, M. & Dameron, C. T. (1999). The *Enterococcus hirae* copper chaperone CopZ delivers copper(I) to the CopY repressor. *FEBS Lett* **445**, 27–30.

Cobine, P. A., George, G. N., Jones, C. E., Wickramasinghe, W. A., Solioz, M. & Dameron, C. T. (2002a). Copper transfer from the Cu(I) chaperone, CopZ, to the repressor, Zn(II)CopY: metal coordination environments and protein interactions. *Biochemistry* **41**, 5822–5829.

Cobine, P. A., Jones, C. E. & Dameron, C. T. (2002b). Role for zinc(II) in the copper(I) regulated protein CopY. *J Inorg Biochem* **88**, 192–196.

Degtyarenko, K. (2000). Bioinorganic motifs: towards functional classification of metalloproteins. *Bioinformatics* **16**, 851–864.

Dixit, V., Bini, E., Drozda, M. & Blum, P. (2004). Mercury inactivates transcription and the generalized transcription factor TFB in the archaeon *Sulfolobus solfataricus*. *Antimicrob Agents Chemother* **48**, 1993–1999.

Edwards, K. J., Bond, P. L., Gihring, T. M. & Banfield, J. F. (2000). An archaeal iron-oxidizing extreme acidophile important in acid mine drainage. *Science* **287**, 1796–1799.

Ettema, T. J., Brinkman, A. B., Tani, T. H., Rafferty, J. B. & Van Der Oost, J. (2002). A novel ligand-binding domain involved in regulation of amino acid metabolism in prokaryotes. *J Biol Chem* **277**, 37464–37468.

Ettema, T. J., Huynen, M. A., de Vos, W. M. & van der Oost, J. (2003). TRASH: a novel metal-binding domain predicted to be involved in heavy-metal sensing, trafficking and resistance. *Trends Biochem Sci* **28**, 170–173.

Gregor, D. & Pfeifer, F. (2001). Use of a halobacterial *bgaH* reporter gene to analyse the regulation of gene expression in halophilic archaea. *Microbiology* **147**, 1745–1754.

Grogan, D. W. (1989). Phenotypic characterization of the archaebacterial genus *Sulfolobus*: comparison of five wild-type strains. *J Bacteriol* **171**, 6710–6719.

Guedon, E. & Helmann, J. D. (2003). Origins of metal ion selectivity in the DtxR/MntR family of metalloregulators. *Mol Microbiol* **48**, 495–506.

Hamza, I., Schaefer, M., Klomp, L. W. & Gitlin, J. D. (1999). Interaction of the copper chaperone HAH1 with the Wilson disease protein is essential for copper homeostasis. *Proc Natl Acad Sci U S A* **96**, 13363–13368.

Harrison, M. D., Jones, C. E., Solioz, M. & Dameron, C. T. (2000). Intracellular copper routing: the role of copper chaperones. *Trends Biochem Sci* **25**, 29–32.

Hochheimer, A., Hedderich, R. & Thauer, R. K. (1999). The DNA binding protein Tfx from *Methanobacterium thermoautotrophicum*: structure, DNA binding properties and transcriptional regulation. *Mol Microbiol* **31**, 641–650.

Hung, I. H., Casareno, R. L., Labesse, G., Mathews, F. S. & Gitlin, J. D. (1998). HAH1 is a copper-binding protein with distinct amino acid residues mediating copper homeostasis and antioxidant defense. *J Biol Chem* **273**, 1749–1754.

Letunic, I., Copley, R. R., Schmidt, S., Ciccarelli, F. D., Doerks, T., Schultz, J., Ponting, C. P. & Bork, P. (2004). SMART 4.0: towards genomic data integration. *Nucleic Acids Res* **32**, D142–D144.

Lutsenko, S. & Kaplan, J. H. (1996). P-type ATPases. *Trends Biochem Sci* **21**, 467.

Mana-Capelli, S., Mandal, A. K. & Arguello, J. M. (2003). *Archaeoglobus fulgidus* CopB is a thermophilic Cu^{2+}-ATPase: functional role of its histidine-rich-N-terminal metal binding domain. *J Biol Chem* **278**, 40534–40541.

Mercer, J. F. (2001). The molecular basis of copper-transport diseases. *Trends Mol Med* **7**, 64–69.

Multhaup, G., Strausak, D., Bissig, K. D. & Solioz, M. (2001). Interaction of the CopZ copper chaperone with the CopA copper ATPase of *Enterococcus hirae* assessed by surface plasmon resonance. *Biochem Biophys Res Commun* **288**, 172–177.

Nies, D. H. (1999). Microbial heavy-metal resistance. *Appl Microbiol Biotechnol* **51**, 730–750.

Ouhammouch, M., Dewhurst, R. E., Hausner, W., Thomm, M. & Geiduschek, E. P. (2003). Activation of archaeal transcription by recruitment of the TATA-binding protein. *Proc Natl Acad Sci U S A* **100**, 5097–5102.

Outten, F. W., Outten, C. E., Hale, J. & O'Halloran, T. V. (2000). Transcriptional activation of an *Escherichia coli* copper efflux regulon by the chromosomal MerR homologue, cueR. *J Biol Chem* **275**, 31024–31029.

Rosenzweig, A. C. (2002). Metallochaperones: bind and deliver. *Chem Biol* **9**, 673–677.

Schelert, J., Dixit, V., Hoang, V., Simbahan, J., Drozda, M. & Blum, P. (2004). Occurrence and characterization of mercury resistance in the hyperthermophilic archaeon *Sulfolobus solfataricus* by use of gene disruption. *J Bacteriol* **186**, 427–437.

Stoyanov, J. V., Hobman, J. L. & Brown, N. L. (2001). CueR (YbbI) of *Escherichia coli* is a MerR family regulator controlling expression of the copper exporter CopA. *Mol Microbiol* **39**, 502–511.

Strausak, D. & Solioz, M. (1997). CopY is a copper-inducible repressor of the *Enterococcus hirae* copper ATPases. *J Biol Chem* **272**, 8932–8936.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Tottey, S., Rondet, S. A., Borrelly, G. P., Robinson, P. J., Rich, P. R. & Robinson, N. J. (2002). A copper metallochaperone for photosynthesis and respiration reveals metal-specific targets, interaction with an importer, and alternative sites for copper acquisition. *J Biol Chem* **277**, 5490–5497.

Walker, J. M., Tsivkovskii, R. & Lutsenko, S. (2002). Metallochaperone Atox1 transfers copper to the NH_2-terminal domain of the Wilson's disease protein and regulates its catalytic activity. *J Biol Chem* **277**, 27953–27959.