Structural and functional characterization of the transcriptional repressor CsoR from Thermus thermophilus HB8

Keiko Sakamoto,† Yoshihiro Agari,† Kazuko Agari,† Seiki Kuramitsu1,2 and Akeo Shinkai1

1RIKEN SPring-8 Center, Harima Institute, 1-1-1 Kouto, Sayo, Hyogo 679-5148, Japan
2Department of Biological Sciences, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan

INTRODUCTION

The copper ion is essential for living organisms as a cofactor for many enzymes; however, it can be very toxic because of its potential to drive oxidation/reduction reactions and to form free radicals (Imlay, 2002; Touati, 2000). Therefore, many organisms have developed mechanisms to control the intracellular copper ion concentration. CsoR, which may be widely distributed in eubacterial genomes, has been functionally identified in Mycobacterium tuberculosis and Bacillus subtilis as a transcriptional repressor that regulates the expression of genes involved in copper homeostasis, including the copper chaperone copZ and copper efflux P-type ATPase copA genes (Liu et al., 2007; Smaldone & Helmann, 2007). CsoR forms a homotetramer and specifically binds a promoter region containing a GC-rich pseudopalindromic sequence (Iwig & Chivers, 2009; Ma et al., 2009a). In the presence of an excess amount of Cu(I), CsoR binds the ion and is then released from the DNA, resulting in the expression of downstream genes and the efflux of excess Cu(I).

Thermus thermophilus HB8, which belongs to the phylum Deinococcus–Thermus, is an extremely thermophilic bacterium isolated from the water at a Japanese hot spring, and grows in an optimum temperature range of 65–72 °C (Oshima & Imahori, 1974). Its genome is composed of 1.85 Mb chromosomal DNA, the 0.26 Mb plasmid pTT27 and the 9.32 kb plasmid pTT8, which encode CopZ, CsoR and the copper efflux P-type ATPase CopA, to repress their expression, while in the presence of approximately an equal amount of copper ion, CsoR was released from the DNA, to allow expression of the downstream genes. Both Cu(I) and Cu(II) ions could bind CsoR, and were effective for transcriptional derepression. Additionally, CsoR could also sense various other metal ions, such as Zn(II), Ag(I), Cd(II) and Ni(II), which led to transcriptional derepression. The copper-binding motif of T. thermophilus CsoR contains C-H-H, while those of most orthologues contain C-H-C. The X-ray crystal structure of T. thermophilus CsoR suggests that a histidine residue in the N-terminal domain is also involved in metal-ion binding; that is, the binding motif could be H-C-H-H, like that of Escherichia coli RcnR, which binds Ni(II)/Co(II). The non-conserved H70 residue in the metal-binding motif of T. thermophilus CsoR is important for its DNA-binding affinity and metal-ion responsiveness.

†These authors contributed equally to this work.

Abbreviations: PDB, Protein Data Bank; RMSD, root mean square deviation; RNAP, RNA polymerase-σ70 holoenzyme; Se-CsoR, CsoR containing selenomethionine.

A supplementary figure, showing gel-filtration analysis of the T. thermophilus wild-type and H70C mutant CsoR proteins, and a supplementary table, showing the oligonucleotides used in this study, are available with the online version of this paper.

The microarray data discussed in this study have been deposited in the NCBI Gene Expression Omnibus (GEO) under accession number GSE19521.
determined by inductively coupled plasma emission spectroscopy is similar to that of *Escherichia coli* (Kondo et al., 2008). In this study, we showed that the TTHA1719 protein is a transcriptional repressor of the copper-sensitive TTHA1718 (copZ)-TTHA1719 (csoR)-TTHA1720 (copA) operon, i.e. that the TTHA1719 protein is an ortholog of CsoR. We also characterized the *T. thermophilus* CsoR structurally and functionally.

**METHODS**

**Overproduction and purification of recombinant CsoR.** The *T. thermophilus* csoR (*TTHA1719*) gene was amplified by genomic PCR using primers P01 and P02 (Supplementary Table S1), and then the amplified fragment was inserted into a TA cloning vector, pT7Blue (Novagen), harboring pET11a-\(\text{ttCsoR}\), was cultured at 37 °C. *E. coli* **\(\text{ttCsoR}\)**. The second codon found in the chromosome, CCC, was added to the supernatant to a final concentration of 2.5 M; then, buffer (pH 7.0) containing 2.5 M \((\text{NH}_4)_2\text{SO}_4\), and the bound protein was eluted with a linear gradient of 2.5–0 M \((\text{NH}_4)_2\text{SO}_4\). The target (**g**) was applied to a RESOURCE ISO column (**1 cm**) at a flow rate of 2 M for 1 h. Ammonium sulfate was added to the supernatant to a final concentration of 2.5 M; then, after the sample had been centrifuged at 23 500 g for 10 min at 4 °C, the resulting supernatant was applied to a RESOURCE ISO column (**GE Healthcare**) pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 50 mM NaCl and 5 mM 2-mercaptoethanol, and disrupted by sonication in ice water. The same volume of buffer without 2-mercaptoethanol preheated at 70 °C was added to the cell lysate, followed by incubation for 10 min at 70 °C, and then ultracentrifugation (200 000 g) for 1 h at 4 °C. Ammonium sulfate was added to the supernatant to a final concentration of 2.5 M; then, after the sample had been centrifuged at 23 500 g for 10 min at 4 °C, the resulting supernatant was applied to a RESOURCE ISO column (**GE Healthcare**) pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 2.5 M \((\text{NH}_4)_2\text{SO}_4\), and the bound protein was eluted with a linear gradient of 2.5–0 M \((\text{NH}_4)_2\text{SO}_4\). The target fractions were collected and desalted by fractionation on a HiPrep 26/10 desalting column (**GE Healthcare**) pre-equilibrated with 20 mM Tris/HCl (pH 8.0). A sample was then applied to a RESOURCE Q column (**GE Healthcare**) pre-equilibrated with the same buffer, and the bound protein was eluted with a linear gradient of 0–0.5 M NaCl. The target fractions were collected and desalted by fractionation on a HiPrep 26/10 desalting column (**GE Healthcare**) pre-equilibrated with 10 mM sodium phosphate buffer (pH 7.0). The sample was then applied to a hydroxyapatite CHT5-I column pre-equilibrated with the same buffer, and the bound protein was eluted with a linear gradient of 10–250 mM sodium phosphate buffer (pH 7.0). The target fractions were collected and desalted and then measured in a Cary 50 (Varian) spectrophotometer. The molar extinction coefficient of the target protein was determined by measuring the absorbance at 280 nm with a molar absorption coefficient of 280 M \(^{-1}\) cm \(^{-1}\) (Kuramitsu et al., 1990). Selenomethionine (SeMet)-containing CsoR (Se-CsoR) was generated using the methionine auxotroph **\(\text{TTTHA1719}\)** (**K. Sakamoto and others**). The data collection and refinement statistics are presented in Table 1.

**X-ray diffraction data collection and structure determination.** A crystal was mounted on a cryoloop and flash-cooled in a nitrogen gas stream at 100 K. Multiple anomalous dispersion (MAD) data were collected at three different wavelengths with a Mar Mosaic 225 charge-coupled device (CCD) detector (Rayonix) using the Structural Genomics Beamline II Synchrotron (BL26B2) at SPring-8 (Hyogo, Japan). The oscillation angle was 0.5 °, the exposure time was 2 s per frame, and the camera distance was 180 mm. All diffraction images were processed using the HKL2000 program suite (Otwinowski & Minor, 1997). Selenium sites were determined with the SOLVE program (Terwilliger & Berendzen, 1999), and the resulting phases were improved with the RESOLVE program (Terwilliger & Berendzen, 1999). The initial model was built with the ARP/wARP program (Perrakis et al., 2001), and further manual model building was performed using Coot (Emsley & Cowtan, 2004). Simulated annealing, energy minimization and B factor refinement were carried out using the CNS program package (Brünger et al., 1998). Cycles of manual modelling and CNS refinement were performed; 10% of the total reflections were randomly chosen for the **R** free sets. The quality of the structure was analysed using PROCHECK (Laskowski et al., 1993) in the CCP4 suite (Collaborative Computational Project, Number 4, 1994) and MolProbity (Lovell et al., 2003). The data collection and refinement statistics are presented in Table 1.

**Monitoring of copper ion binding by UV absorption spectroscopy.** CsoR proteins (1.0–1.6 mM monomer) were dialysed against a buffer containing 20 mM MES-NaOH (pH 7.0) and 0.5 M NaCl, and the proteins were dialysed to 20.4 M with a buffer containing 20 mM MES-NaOH (pH 7.0) and 0.2 M NaCl. Then, 5 μM CuCl₂ solution was added to 245 μM of the protein solution, followed by incubation at 55 or 25 °C for 6 min. Subsequently, 1 μl of 50 mM DTT or H₂O₂ was added, and then after 5 min, the **A**₂₈₀ was measured.

**BIacore biosensor assay.** A DNA fragment (0.1 mM), biotinylated at the 5' end of one strand, was diluted to 50 nM with a buffer containing 10 mM HEPES-NaOH (pH 7.4), 0.5 M NaCl, and the proteins were diluted to 20.4 M with a buffer containing 20 mM MES-NaOH (pH 7.0) and 0.2 M NaCl. Then, 5 μM CuCl₂ solution was added to 245 μM of the protein solution, followed by incubation at 55 or 25 °C for 6 min. Subsequently, 1 μl of 50 mM DTT or H₂O₂ was added, and then after 5 min, the **A**₂₈₀ was measured.

**In vitro transcription assay: preparation of templates.** The upstream region of the *TTHA1718* gene (copZ) corresponding to

(see below) was overexpressed and purified by the same process as for the wild-type native protein.

**Crystallization.** Crystallization of Se-CsoR was performed by the sitting-drop vapour diffusion method by equilibrating a mixture of 0.7 μl of a protein solution (10.4 mg ml⁻¹) with an equal volume of a reservoir solution containing 4.25 M sodium formate and 5%, w/v, (+/-)1-2-methyl-2,4-pentanediol against 0.1 ml of the reservoir solution at 20 °C. Crystals grew within 7 days to maximum dimensions of 50 x 50 x 50 μm.
Table 1. X-ray data collection and refinement statistics

Values in parentheses are for the highest-resolution shell.

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* Rmerge = ΣhIh − <Ih>/Σh Ih
† Rwork = Σ(hFh − |Fh|)/Σh Ih, where Ih is the mean intensity of reflection h.
‡ Rmerge is the R factor calculated using 10% of the data that were excluded from the refinement.
§ Calculated with MolProbity.

positions 1614 339–1614 420 of the chromosomal DNA was amplified by genomic PCR using primers P03 and P04 (Supplementary Table S1), and inserted into a TA cloning vector, pT7Blue (Novagen). The 90 bp BamHI–EcoRI fragment of the plasmid was ligated into pUC19 (Novagen). Construction of a plasmid containing the E. coli consensus promoter named full con has been described previously (Shinkai et al., 2007). Using each plasmid as the template, PCR was performed with primers P05 and P06 (Supplementary Table S1) to prepare a template. The amplified fragments were excised from a 0.8 %, w/v, agarose gel, extracted with phenol and ether, and then precipitated with ethanol. The DNA fragments were used for the assay below.

In vitro transcription assay: run-off transcription. The assays were performed in 15 µl reaction mixtures. The reaction mixture was composed of buffer A (50 mM glycine-NaOH, pH 8.5, 18 mM MgCl₂, 50 mM KCl) with or without 10 mM DTT, 0.3 mM each rNTP, 5.55 × 10⁶ Bq [α-³²P]dCTP (MP Biomedicals), 0.2 µM template DNA, 50 nM RNA polymerase-α DNA polymerase (RNAP) from T. thermophilus HB8 purified as described previously (Vassyleva et al., 2002), and 50 µg BSA ml⁻¹. The template DNA was pre-incubated with or without CsoR, in the presence or absence of a metal salt with or without DTT, at 37 °C for 5 min. The RNAP was then added, and the mixture was incubated for a further 5 min. Transcription was initiated by the addition of 5.55 × 10⁶ Bq [α-³²P]dCTP and unlabelled rNTPs. After a further 10 min incubation, the reaction was stopped by the addition of 11 µl of a stop mix composed of 20 mM EDTA, 96 %, w/v, deionized formamide, 0.01 %, w/v, bromophenol blue and 0.01 %, w/v, xylene cyanol. The sample was analysed on a 10 %, w/v, polyacrylamide gel containing 8 M urea, with visualization by autoradiography.

Identification of the in vitro transcriptional start site. RNA was synthesized at 55 °C for 20 min, in a 0.1 ml reaction mixture composed of buffer A, 0.1 mM EDTA, 0.2 µM template DNA, 0.1 µM T. thermophilus RNAP, 0.3 mM each rNTP and 50 µg BSA ml⁻¹. The sample was treated with 5 U RNase-free DNase I (Takara Bio) at 37 °C for 10 min. After the DNase had been inactivated by heat treatment followed by phenol extraction, the RNA was precipitated with 2-propanol in the presence of 1 % glycogen. The sample was dissolved in 0.1 ml H₂O and then precipitated with ethanol. Using the RNA as a template and P06 (Supplementary Table S1) as a primer, cDNA was synthesized with 2.5 U AMV Reverse Transcriptase XL (Takara Bio) in a 20 µl reaction mixture containing 0.5 mM each dNTP and 9.17 × 10⁵ Bq [α-³²P]dCTP (MP Biomedicals) at 42 °C for 20 min. The reaction was stopped by the addition of 15 µl of the stop mix. The nucleotide sequence of the template DNA was determined by the dideoxy-mediated chain-termination method (Sanger et al., 1977) with primer P06, using T7 DNA polymerase (GE Healthcare). Samples were analysed on an 8 %, w/v, polyacrylamide gel containing 8 M urea, with visualization by autoradiography.

Disruption of the T. thermophilus csoR gene. Plasmid pGEM-ΔcsoR used for disruption carries the downstream region (positions

http://mic.sgmjournals.org
1 615 414–1 614 891 of the chromosomal DNA of the csoR gene in the opposite direction, followed by the thermostable kanamycin-resistance marker (HTK) gene and the upstream region (positions 1 614 616–1 614 120 of the chromosomal DNA) of the csoR gene in the opposite direction, as described by Hashimoto et al. (2001). The HTK gene was amplified by PCR from a plasmid, pJKH3 (Hoseki et al., 1999), and then the amplified fragments were ligated into a TA cloning vector, pT7Blue (Novagen).

The 500 bp fragment containing the downstream or upstream region of csoR was amplified by genomic PCR, using the primers listed in Supplementary Table S1, and then each amplified fragment was ligated into pTA cloning vector, pT7Blue (Novagen). The Hpal–XbaI fragment containing the downstream region of csoR and the PstI–EcoRV fragment containing the upstream region of csoR were ligated into pGEM-HTK-Xba to construct pGEM-HTK-Xba. The 500 bp fragment containing the downstream or upstream region of csoR was amplified by genomic PCR, using the primers listed in Supplementary Table S1, and then each amplified fragment was ligated into a TA cloning vector, pT7Blue (Novagen).

DNA microarray analysis: culture conditions for T. thermophilus HB8. T. thermophilus HB8 was precultured at 70 °C for 16 h in 3 ml of a rich medium (TT broth) (Agari et al., 2008). The cells (1 ml) were inoculated into 250 ml of a synthetic medium prepared as described previously (Agari et al., 2008) and then cultivated at 70 °C. After 8 h [OD600 = 0.4 (1 cm path, SmartSpec Plus, Bio-Rad)], i.e., in the exponential growth phase, cells were collected from 50 ml culture medium, and CuSO4 or ZnSO4 was added to the remaining medium to a final concentration of 1.25 mM for CuSO4 or 1 mM for ZnSO4 after which cultivation was continued. After 30 min, cells were collected from 50 ml culture medium.

DNA microarray analysis: CDNA synthesis, hybridization and data analysis. Crude RNA was extracted from each lot of cells, and then CDNA was synthesized, followed by fragmentation and labelling with biotin–dideoxy–UTP, as described previously (Agari et al., 2008). The 3′ terminal-labelled CDNA was hybridized to a TTHB8401a520105FS GeneChip (Affymetrix), and then the array was washed, stained and scanned as described previously (Agari et al., 2008). The raw intensities for three independently cultivated wild-type and ΔcsoR strains were summarized as 2266 ORFs using GeneChip Operating Software version 1.4 (Affymetrix).

(i) Expression analysis at the ORF level. The datasets were normalized through the following three normalization steps using the Subio Platform (Subio Inc.), i.e., data transformation (set measurements of less than 0.01–0.01), followed by per chip normalization (normalized with respect to the median) and per gene normalization (normalized using the mean value of the wild-type data or the mean value of the data before the addition of a metal as control samples). The t test P value for the observed differences in the normalized intensities between the wild-type and the ΔcsoR strains or between pre- and post-addition of a metal ion was calculated using the Subio Platform, and then from the obtained value, the false discovery rate (q value) (Storey & Tibshirani, 2003) was calculated using R (http://www.R-project.org).

(ii) Expression analysis at the probe level. Three perfect match probe intensities for pre- and post-addition of CuSO4 were simultaneously quantile-normalized (Bolstad et al., 2003). In order to determine the expression differences between pre- and post-addition of CuSO4, the mean values of the three probe intensities for post-addition of CuSO4 were each divided by the pre-addition values. Then, the Wilcoxon signed-rank test was applied with a ±100 bp window positioned at the centre of each probe, which gave the P value for the centre probe (Cawley et al., 2004). Data processing, statistical analysis and data visualization were performed using R and a bioconductor (Gentleman et al., 2004).

Constructions of the H70C mutant gene. Site-directed mutagenesis was performed on plasmid pET11a-ttCsoR to introduce C208 to T and A209 to G mutations of the csoR gene, changing the codon CAC for His70 to TGC for Cys, using a QuickChange site-directed mutagenesis kit (Stratagene) with primers P07 and P08 (Supplementary Table S1).

Other methods. N-terminal sequence analysis of proteins was performed with a protein sequencer (Procise HT, Applied Biosystems). Gel-filtration chromatography to determine the molecular mass of T. thermophilus CsoR was performed on a Superdex 75 HR 10/30 column (GE Healthcare). A BLAST search was performed on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST). The percentage identity and similarity to the amino acid sequence of T. thermophilus CsoR were determined using the EMBOSS pairwise alignment algorithms program (http://www.ebi.ac.uk/Tools/emboss/align/). A nucleotide sequence motif search was performed with the GENETYX ver. 8.0 program (GENETYX).

Database accession number. The atomic coordinates and structure factors for CsoR have been deposited in the Protein Data Bank (PDB) under accession number 3AAI.

RESULTS

Initial characterization of the TTHA1719 (CsoR) protein

The TTHA1719 ORF encodes 94 amino acid residues (NCBI accession no. YP_144985.1) with a predicted molecular mass of 10.9 kDa. A BLAST search revealed that the TTHA1719 protein belongs to the DUP156 superfamily like the M. tuberculosis and B. subtilis CsoR proteins, which show homology to the TTHA1719 protein with e values of 4e-17 and 7e-13, identities of 29.8 and 31.4 %, and similarities of 46.0 and 50.0 %, respectively (Fig. 1). Of the three residues C36, H61 and C65 that are involved in copper ion binding by M. tuberculosis CsoR (Liu et al., 2007), two are conserved in the TTHA1719 protein, i.e. C41 and H66 (Fig. 1) (see below). Other conserved residues include Y40 and E86, which have been found to be involved in allosteric modulation of the DNA-binding domain in response to bound copper ions (Liu et al., 2007; Ma et al., 2009a). The TTHA1719 protein was over-expressed in E. coli, and the recombinant protein was purified from a cell lysate. The lysate, which was resistant to treatment at 70 °C for 10 min, was fractionated with ammonium sulfate. This was followed by hydrophobic, anion-exchange, hydroxyapatite and gel-filtration column chromatography. A protein with a purity of >95 % on SDS-PAGE was obtained. The N-terminal amino acid
sequence of the purified protein was P-H-S-H (data not shown), indicating that the N-terminal methionine had been deleted. The molecular mass of the TTHA1719 protein, as determined by gel-filtration chromatography, was ~40 kDa (Supplementary Fig. S1), suggesting that it exists as a stable homotetramer in solution.

We found that the TTHA1719 protein is a copper-sensing transcriptional repressor (see below); therefore, we named this protein *T. thermophilus* CsoR, as it is structurally and functionally similar to the transcriptional regulators from *M. tuberculosis* and *B. subtilis* (Liu et al., 2007; Smaldone & Helmann, 2007).

**X-ray crystal structure of *T. thermophilus* CsoR**

The 3D crystal structure of CsoR was determined at a resolution of 2.1 Å, with crystallographic factors of 24.4 and 28.8 %, respectively (Table 1). An asymmetrical unit of the crystal comprised one homotetramer of CsoR. The tetrameric structure was composed of two dimers, i.e. chains AB and CD (Fig. 2a). This assembly was predicted from the PISA (Protein Interfaces, Surfaces and Assemblies service) at the European Bioinformatics Institute (http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html) (Krissinel & Henrick, 2007).

Each CsoR monomer contains three α-helices, i.e. α1 (P10–L34), α2 (C41–D69) and α3 (V80–L91), for chain A. The Cα root mean square deviation (RMSD) values between chains A and B and between chains C and D were 1.24 and 1.26 Å, respectively (Fig. 2b). Large conformational differences between the monomers were found around the putative copper ion-binding site (see below; Fig. 2e, f). Note that there are several disordered main-chain regions that were not included in the model: M1–L6, V71–G78 and Y93–R94 of chain A; M1–S4, T73–E82 and K92–R94 of chain B; M1–L6, V71–D79 and Y93–R94 of chain C; and M1–S4, A72–E82 and Y93–R94 of chain D (Fig. 2a). The coordinates of the side chains of K11, D79, E85 and E86 of chain A; E37, K38, E85 and E86 of chain B; K11, E14, E82, E85, K92 of chain C; and E37, K38 and E85 of chain D could not be determined due to their poor electron densities. The structure is most closely related to that of *M. tuberculosis* CsoR (Liu et al., 2007), the Z-score and RMSD being 97.7 and 1.6 Å for chain A, and 89.1 and 1.5 Å for chain B, respectively (Holm & Sander, 1998) (Fig. 2b). α1 and α2 from one monomer and those from another of *T. thermophilus* CsoR form a similar standard antiparallel four-helix bundle to that of *M. tuberculosis* CsoR. The interaction surface area at the interface of chains A and B, calculated with the ArealMol program (Collaborative Computational Project, Number 4, 1994), is ~2073 Å², which accounts for ~30 % of the total surface area of chain A. The structures of the two CsoR dimers are similar, as shown by the RMSD value of 0.15 Å for the corresponding Cα atoms. The C-terminal region of α2 of chain A and that of chain B are packed against α3 of chain D and that of chain C, respectively (Fig. 2a). L63, L67, V71, I83, V84, L87, M88 and L91 are involved in the formation of a hydrophobic core on the surface (Fig. 2c). The interaction surface area at the interface of chains AB and CD, calculated with the ArealMol program, is ~1265 Å², which accounts for ~14 % of the total surface area of the chains in the AB dimer.

Clear electron densities corresponding to copper ions could not be detected, unlike in the case of *M. tuberculosis* CsoR. We predicted the copper ion-binding site of *T. thermophilus* CsoR by superposition on the crystal structure of *M. tuberculosis* CsoR (Fig. 2d–f). It looks as if the C-terminal portion of the α2 helix of *M. tuberculosis* CsoR bends toward the copper ion, but does not in the case of *T. thermophilus* CsoR (Fig. 2d–f). C41, H66 and H70 of *T. thermophilus* CsoR, corresponding to the copper-binding residues of *M. tuberculosis* CsoR, i.e. C36, H61 and C65, are close to the copper ion. In addition to these residues, H5 of *T. thermophilus* CsoR, for which there is no

![Fig. 1. Amino acid sequence alignment of the *T. thermophilus* (Tt), *M. tuberculosis* (Mt) and *B. subtilis* (Bs) CsoR proteins, and *E. coli* (Ec) RcnR. The sequences were aligned using CLUSTAL W2 (Larkin et al., 2007). The secondary structure was predicted with DSSP (Kabsch & Sander, 1983), and the figure was generated with ESPript 2.2 (Gouet et al., 2003). The percentage identities (id.) and similarities (sim.) to Tt CsoR are indicated on the right. The positions of the conserved copper-coordinating residues of CsoR are indicated by asterisks.](image_url)
Corresponding residue in *M. tuberculosis* CsoR (Fig. 1), is close to the copper ion; therefore, the residue might be involved in the binding of a copper ion (Fig. 2e). H5 might correspond to H3 of *E. coli* RcnR (Fig. 1), which is involved in the binding of Ni(II)/Co(II) ions (Iwig *et al.*, 2008; Ma *et al.*, 2009b).

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**Fig. 2.** X-ray crystal structure of *T. thermophilus* CsoR. (a) Ribbon diagrams of the *T. thermophilus* CsoR tetramer. Chains A, B, C and D are coloured red, blue, grey and white, respectively. The putative position of the copper ion is indicated by an orange sphere. (b) Superpositioning of the Cα structures of *T. thermophilus* CsoR chains A (red) and B (grey), and *M. tuberculosis* CsoR (green). (c) Stereoview of the dimer–dimer interface of CsoR. Chains B (blue) and C (grey) are shown. The side chains of the indicated residues are depicted as stick models. Stereoviews around the copper ion-binding sites of *M. tuberculosis* CsoR (Liu *et al.*, 2007) (d), the corresponding sites around C41 of chain A (e) and those around C41 of chain B (f) of *T. thermophilus* CsoR. Copper ions are indicated by orange spheres. These diagrams were generated with the program PyMol (http://pymol.sourceforge.net/).
Copper ion-binding ability of *Thermus thermophilus* CsoR

We investigated the copper ion-binding ability of *T. thermophilus* CsoR by UV absorption spectroscopy of thiolate–copper coordination bonds, as in the case of *M. tuberculosis* and *B. subtilis* CsoR proteins (Liu et al., 2007; Ma et al., 2009a), in the presence or absence of 0.2 mM DTT as a reductant at both 55 and 25 °C. The A$_{240}$ of CsoR increased with increasing concentrations of CuCl$_2$ in both the presence and the absence of DTT (Fig. 3a). CuCl$_2$ was more effective in the presence of DTT than in its absence. Basically, the same results were obtained at 55 and 25 °C. We confirmed that 40 μM Cu(II) ion was immediately converted to Cu(I) on the addition of 0.2 mM DTT, and that more than 90% of the copper ions were still in the form of Cu(I) after 5 min at 55 °C, by using bathocuproinedisulfonic acid (BCS), which forms a Cu(I)(BCS)$_2$ complex exhibiting absorbance at 483 nm (data not shown). These results indicate that *T. thermophilus* CsoR can bind both Cu(I) and Cu(II) through a thiolate–copper coordination bond, and that it preferentially binds Cu(I). The C41 residue in the copper-binding motif may bind the copper ion. We could not monitor the absorbance of a CsoR protein solution containing more than 20 μM CuCl$_2$ without DTT because a precipitate appeared under the conditions used.

DNA-binding ability of *Thermus thermophilus* CsoR

According to the genome analysis results, csoR forms an operon with the TTHA1718 (putative metal-binding protein, copZ) and TTHA1720 (putative cation ATPase, copA) genes (NCBI accession no. NC_006461) (Fig. 4b). Upstream of the copZ gene, a pseudopalindromic sequence is similar to the binding sites of the *M. tuberculosis* and *B. subtilis* CsoR proteins was found (Fig. 4b). The transcription start site of the copZ-csoR-copA operon determined from the in vitro transcript was in the palindromic sequence, and that the promoter overlaps the sequence (Fig. 4a, b). We investigated the binding ability of *T. thermophilus* CsoR with respect to the palindromic sequence by means of BIACore analysis. A dsDNA containing the predicted binding site, 5’-GGCGGTACCCACCCACCTGGGGTGGGTTAGGAG-3’, was immobilized on the streptavidin surface of a sensor chip, through biotin conjugated at the 5’ end of one of the DNA strands. *T. thermophilus* CsoR was injected over the DNA surface at 25 °C, and it was found that it bound the DNA (Fig. 5a). CsoR did not bind the dsDNA derived from f1 MR phage, 5’-AGCCGAATTTTTAGACTGATGGCATGTGA-3’ which binds MutL protein (Blackwell et al., 2001), suggesting that *T. thermophilus* CsoR binds specifically to the identified binding site. Thus, the palindromic sequence located upstream of the copZ gene is possibly a CsoR-binding site. Kinetic constants for the binding with the aforementioned palindromic sequence were determined from the sensograms obtained at various concentrations of CsoR, as shown in Table 2.

To find other possible *T. thermophilus* CsoR-binding sites, we searched for potential binding sites in the whole genome of *T. thermophilus* HB8 by using a consensus motif, 5’-CCCCACCCNNNNGGGTTGGG-3’ (N=G, A, T or C), as a query. However, sequences similar to that of the putative CsoR-binding site were not found.

**Effects of *T. thermophilus* CsoR on transcription in vitro**

A DNA fragment from the upstream region of copZ containing the CsoR-binding site identified above was cloned and used as a template for in vitro run-off transcription assays. We found that the DNA fragment was transcribed by *T. thermophilus* RNAP, and that the transcription reaction was repressed in the presence of *T. thermophilus* CsoR (Fig. 6a). CsoR had no effect on the transcription of a DNA fragment containing the *E. coli* consensus promoter (Fig. 6b) in either the presence or the absence of DTT, which also indicates that CsoR specifically binds the promoter of the copZ-csoR-copA operon. We investigated the effects of both Cu(II) and Cu(I) ions on the activity of CsoR. CsoR lost the ability to repress transcription in the presence of an approximately equimolar amount of CuCl$_2$ in both the presence and the absence of 10 mM DTT (Fig. 6a). Thus, the results indicate that both Cu(II) and Cu(I) ions can bind *T. thermophilus* CsoR, and are capable of converting this protein into a DNA-unbound form. We found that other metal ions, roughly in the order of Zn(II) and Cd(II) >Ag(I) and Ni(II) >Co(II) >Fe(III) >Pb(II), could also derepress

**Fig. 3.** Copper ion binding to the *T. thermophilus* wild-type (a) and H70C mutant (b) CsoR proteins. Different amounts of CuCl$_2$ were mixed with 20 μM CsoR monomer in 20 mM MES (pH 7.0) and 0.2 M NaCl at 55 °C (circles) or 25 °C (triangles) in the absence (filled symbols) or presence (open symbols) of 0.2 mM DTT. The A$_{240}$ was plotted against the total copper ion concentration.
transcription (Fig. 6c). We confirmed that these ions at these concentrations did not affect the in vitro transcription activity of T. thermophilus RNAP (data not shown). These results suggest that CsoR is a transcriptional repressor that responds not only to copper but also to other metal ions.

Activity of T. thermophilus CsoR in vivo

The altered mRNA expression of the T. thermophilus wild-type strain after cultivation for 30 min in the presence of 1.25 mM CuSO₄ was investigated by DNA microarray analysis at both the ORF (Table 3) and probe (Fig. 7a) levels. Expression of the copZ-csoR-copA operon was found to be significantly increased after cultivation in the presence of CuSO₄, which is consistent with the results of in vitro transcription assays. Additionally, expression of the downstream genes, i.e. TTHA1721–TTHA1733, with the exception of TTHA1729, was remarkably increased after cultivation in the presence of CuSO₄. In order to confirm the target genes of CsoR, altered expression of these genes in the ΔcsoR strain, in which csoR is replaced by the HTK gene, was investigated (Table 3). As expected, expression of copZ was increased in the ΔcsoR strain. The decreased expression of copA is perhaps due to a negative effect of insertion of the HTK gene upstream of the gene. Unlike the wild-type strain, the ΔcsoR strain showed a growth defect after the addition of CuSO₄ (Fig. 8), which might be due to the accumulation of excess copper ions in the strain owing to decreased expression of copA. Expression of the TTHA1721–TTHA1732 genes decreased or was not significantly altered in the ΔcsoR strain; furthermore, expression of all these genes except TTHA1729 increased

![Fig. 4](image-url)

**Fig. 4.** (a) Identification of the in vitro transcription start site. The RNA transcribed by T. thermophilus RNAP (lane 5) from the genes containing the sequence upstream of T. thermophilus copZ was reverse-transcribed, and the nucleotide sequence of the template DNA was determined by the dideoxy-mediated chain-termination method (lanes 1–4). After the reaction, samples were analysed by PAGE followed by autoradiography. The 3' terminus of the cDNA is indicated by an asterisk. (b) Schematic representation of the T. thermophilus copZ-csoR-copA locus and nucleotide sequence of the copZ promoter region (T.th PcopZ). The genome positions in the chromosome are indicated. The sequence is aligned with the binding sites of the CsoR proteins from M. tuberculosis (M.tb PcsoR) and B. subtilis (B.su PcopZ). Arrows indicate pseudopalindromic sequences. Transcription start sites are designated by upper-case type. Putative −35 and −10 promoter elements are underlined with broken and solid lines, respectively.

![Fig. 5](image-url)

**Fig. 5.** BIAcore biosensor analysis of the CsoR–DNA interaction. A dsDNA containing the upstream sequence of the T. thermophilus copZ gene was immobilized to yield about 450 response units (RU) on the sensor chip, and then the wild-type (a) or H70C mutant (b) T. thermophilus CsoR was injected over the DNA surface at concentrations of 1, 0.5, 0.4, 0.3 and 0.2 μM monomer. Representative sensorgrams, minus the bulk refractive index background, were recorded and normalized with respect to the baseline of 0 RU, as described in Methods.
in the ΔcsoR strain in the presence of CuSO₄, suggesting that they are not targets of CsoR (Table 3, Fig. 7b). They might be regulated by unknown transcriptional regulator(s), including a putative response regulator, TTHA1722, and a putative sensor histidine kinase, TTHA1723, which are also non-CsoR-regulated genes. Expression of TTHA1733 (copB), another possible ATP-dependent transmembrane copper ion pump gene, was not drastically altered by the addition of the copper ion or csoR gene disruption, suggesting that this gene is not a direct target of CsoR.

Since T. thermophilus CsoR effectively lost the ability to repress in vitro transcription in the presence of the Zn(II) ion (Fig. 6c), we investigated the altered mRNA expression of the T. thermophilus wild-type strain after cultivation in the presence of 1 mM ZnSO₄ (Table 3). Expression of the copZ-csoR-copA operon was found to be significantly increased after cultivation in the presence of ZnSO₄, which is consistent with the results of in vitro transcription assays.

The T. thermophilus HB8 strain showed a growth defect after the addition of 1 mM ZnSO₄ (Fig. 8). The growth of the ΔcsoR strain was more affected than that of the wild-type strain (Fig. 8), suggesting that excess zinc ions are accumulated in the strain owing to decreased expression of copA, which may efflux zinc ions.

### Effects of the H70C mutation on the activity of T. thermophilus CsoR

In M. tuberculosis CsoR, three residues, i.e. C36, H61 and C65, are involved in copper ion binding, and these residues are conserved in most orthologues from other species (Liu et al., 2007). However, the corresponding residues in T. thermophilus CsoR are C41, H66 and H70; that is, C65 of M. tuberculosis CsoR is not conserved in T. thermophilus CsoR (Figs 1 and 2d–f), as in the case of Deinococcus CsoR (Liu et al., 2007). In order to determine the importance of the residue with respect to the activity of T. thermophilus

#### Table 2. Kinetic constants for the interaction of CsoR with DNA

The kinetic constants for T. thermophilus CsoR were determined using a BIACore system, as described in Methods and in the legend to Fig. 5. Each value is the mean ± SD of the injection series.

<table>
<thead>
<tr>
<th>CsoR</th>
<th>$k_{on}$ (M⁻¹ s⁻¹)</th>
<th>$k_{off}$ (s⁻¹)</th>
<th>$K_d$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>$(6.5 \pm 1.3) \times 10^4$</td>
<td>$(9.5 \pm 5.5) \times 10^{-4}$</td>
<td>$(1.4 \pm 0.6) \times 10^{-8}$</td>
</tr>
<tr>
<td>H70C mutant</td>
<td>$(1.0 \pm 0.2) \times 10^4$</td>
<td>$(9.7 \pm 7.3) \times 10^{-7}$</td>
<td>$(9.0 \pm 5.2) \times 10^{-11}$</td>
</tr>
</tbody>
</table>

Fig. 6. In vitro run-off transcription assays. (a) The assay was performed with a template containing the upstream sequence of T. thermophilus copZ in the absence (−) or presence of 4 μM wild-type (WT) or H70C mutant (M) T. thermophilus CsoR monomer with the indicated amounts of CuCl₂, in the presence or absence of 10 mM DTT. Lane 1, [α-³²P]dCTP-labelled MspI fragments of pBR322. (b) The assay was performed with a template containing the E. coli consensus promoter designated full con in the absence (−) or presence of 4 μM wild-type (WT) or H70C mutant (M) T. thermophilus CsoR monomer in the presence or absence of 10 mM DTT. Lane 1, [α-³²P]dCTP-labelled MspI fragments of pBR322. (c) The assay was performed with a template containing the upstream sequence of T. thermophilus copZ in the presence of 4 μM wild-type (WT) or H70C mutant (M) T. thermophilus CsoR monomer with 20 μM ZnSO₄ (Zn), AgNO₃ (Ag), Pb(OAc)₂ (Pb), FeCl₃ (Fe), CoCl₂ (Co), NiSO₄ (Ni) or CdSO₄ (Cd), in the absence of DTT. After the reaction, equivalent volumes of samples were analysed by PAGE followed by autoradiography.
Table 3. Altered expression of genes determined by DNA microarray analysis at the ORF level

WT_Cu, altered expression in the wild-type strain after cultivation for 30 min in the presence of 1.25 mM CuSO₄; ΔcsoR, expression in the ΔcsoR strain relative to that in the wild-type; ΔcsoR_Cu, altered expression in the ΔcsoR strain after cultivation for 30 min in the presence of 1.25 mM CuSO₄; WT_Zn, altered expression in the wild-type strain after cultivation for 30 min in the presence of 1.0 mM ZnSO₄. The arrangement of the genes can be seen at the NCBI Entrez Genome website (http://www.ncbi.nlm.nih.gov/sites/entrez?Db=genome&Cmd=ShowDetailView&TermToSearch=530).

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT_Cu expression (q value)</th>
<th>ΔcsoR expression (q value)</th>
<th>ΔcsoR_Cu expression (q value)</th>
<th>WT_Zn expression (q value)</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTHA1718 (copZ)</td>
<td>4.81 (0.01)</td>
<td>6.02 (0.02)</td>
<td>2.59 (0.02)</td>
<td>9.98 (0.00)</td>
<td>Heavy metal-binding protein</td>
</tr>
<tr>
<td>TTHA1719 (csoR)</td>
<td>6.51 (0.01)</td>
<td>22</td>
<td>9.91 (0.00)</td>
<td>9.91 (0.00)</td>
<td>Copper-sensing transcriptional repressor</td>
</tr>
<tr>
<td>TTHA1720 (copA)</td>
<td>7.60 (0.00)</td>
<td>0.10 (0.02)</td>
<td>0.38 (0.10)</td>
<td>11.0 (0.00)</td>
<td>Copper-transporting ATPase</td>
</tr>
<tr>
<td>TTHA1721</td>
<td>11.3 (0.00)</td>
<td>0.38 (0.02)</td>
<td>16.0 (0.01)</td>
<td>6.34 (0.00)</td>
<td>Hypothetical protein</td>
</tr>
<tr>
<td>TTHA1722</td>
<td>12.4 (0.00)</td>
<td>0.38 (0.02)</td>
<td>11.1 (0.01)</td>
<td>4.94 (0.00)</td>
<td>Putative response regulator (CopR)</td>
</tr>
<tr>
<td>TTHA1723</td>
<td>7.93 (0.02)</td>
<td>0.64 (0.16)</td>
<td>5.06 (0.01)</td>
<td>3.80 (0.00)</td>
<td>Sensor histidine kinase (CopS)</td>
</tr>
<tr>
<td>TTHA1724</td>
<td>29.5 (0.00)</td>
<td>0.90 (0.34)</td>
<td>32.7 (0.01)</td>
<td>3.17 (0.01)</td>
<td>Hypothetical protein</td>
</tr>
<tr>
<td>TTHA1725</td>
<td>23.7 (0.00)</td>
<td>0.66 (0.08)</td>
<td>19.1 (0.01)</td>
<td>1.99 (0.01)</td>
<td>S-layer repressor</td>
</tr>
<tr>
<td>TTHA1726</td>
<td>14.1 (0.01)</td>
<td>0.83 (0.25)</td>
<td>8.68 (0.01)</td>
<td>1.77 (0.01)</td>
<td>Hypothetical protein</td>
</tr>
<tr>
<td>TTHA1727</td>
<td>11.6 (0.00)</td>
<td>1.12 (0.26)</td>
<td>6.35 (0.01)</td>
<td>2.52 (0.00)</td>
<td>Conserved hypothetical protein</td>
</tr>
<tr>
<td>TTHA1728</td>
<td>13.2 (0.00)</td>
<td>1.38 (0.04)</td>
<td>3.67 (0.01)</td>
<td>2.08 (0.04)</td>
<td>Putative methyltransferase</td>
</tr>
<tr>
<td>TTHA1729</td>
<td>0.85 (0.15)</td>
<td>1.07 (0.30)</td>
<td>1.03 (0.26)</td>
<td>1.19 (0.14)</td>
<td>Hypothetical protein</td>
</tr>
<tr>
<td>TTHA1730</td>
<td>15.5 (0.01)</td>
<td>0.86 (0.31)</td>
<td>5.24 (0.02)</td>
<td>1.11 (0.22)</td>
<td>Hypothetical protein</td>
</tr>
<tr>
<td>TTHA1731</td>
<td>9.80 (0.00)</td>
<td>1.11 (0.14)</td>
<td>3.09 (0.01)</td>
<td>1.19 (0.03)</td>
<td>Hypothetical protein</td>
</tr>
<tr>
<td>TTHA1732</td>
<td>6.89 (0.00)</td>
<td>1.15 (0.15)</td>
<td>2.68 (0.01)</td>
<td>1.63 (0.01)</td>
<td>Hypothetical protein</td>
</tr>
<tr>
<td>TTHA1733</td>
<td>2.16 (0.01)</td>
<td>1.67 (0.04)</td>
<td>0.85 (0.18)</td>
<td>2.01 (0.00)</td>
<td>Copper-transporting ATPase, P-type (CopB)</td>
</tr>
</tbody>
</table>

Fig. 7. Altered expression of the copZ-csoR-copA operon and its downstream genes after addition of CuSO₄, determined by DNA microarray analysis. Expression in the T. thermophilus HB8 wild-type (a) and ΔcsoR (b) strains after cultivation for 30 min in the presence of 1.25 mM CuSO₄ relative to no addition of CuSO₄ was analysed at the probe level, and is presented as the log₂-transformed normalized intensity of each probe. Only the results for (+) strands are shown. The P value range is indicated by colours, i.e. P<10⁻⁴ (red), 10⁻⁴ <P<10⁻³ (black), and 10⁻³ <P (grey), respectively. ‘TTHA’-omitted gene names are indicated at the top of each panel.
CsoR, an H70C mutant CsoR (CsoR_H70C) was constructed, and its properties were assessed in vitro. Gel-filtration analysis showed that CsoR_H70C forms a stable homotetramer, as in the case of the wild-type (Supplementary Fig. S1). CsoR_H70C could bind the Cu(II) ion to almost the same level as the wild-type CsoR; however, its affinity for Cu(I) was decreased compared with the wild-type (Fig. 3a, b). Basically, the same results were obtained at 55 and 25 °C (Fig. 3b). CsoR_H70C could bind to the CsoR-binding site located upstream of the copZ gene (Fig. 5b), but not to DNA derived from the f1 MR phage (data not shown), indicating that it specifically binds the promoter of the copZ-csoR-copA operon. Interestingly, the dissociation constant was about three orders of magnitude lower than that of the wild-type, which was mainly due to the lower dissociation rate constant (Table 2). CsoR_H70C repressed transcription of the DNA fragment containing the upstream sequence of copZ (Fig. 6a), but did not act on the E. coli consensus promoter, as in the case of wild-type CsoR (Fig. 6b). Cu(II) could derepress transcription, although the effect was less than that on the wild-type CsoR (Fig. 6b). Cu(II) could derepress transcription, although the effect was less than that on the wild-type CsoR (Fig. 6a). Interestingly, the responsiveness to other metal ions also differed between the wild-type and CsoR_H70C proteins in vitro (Fig. 6c). These results indicate that H70, but not a cysteine residue, is important for the DNA-binding affinity and metal ion responsiveness of T. thermophilus CsoR.

**DISCUSSION**

In this study, we identified T. thermophilus CsoR as a transcriptional regulator of the copper-sensitive operon copZ-csoR-copA, both in vitro and in vivo. The crystal structure of T. thermophilus CsoR is similar to that of M. tuberculosis CsoR. Thus, this thermophilic bacterium has a similar copper homeostasis system to those of the mesophiles M. tuberculosis and B. subtilis. Interestingly, T. thermophilus CsoR senses both Cu(II) and Cu(I), and other metal ions such as Zn(II), Cd(II), Ag(I), Ni(II) and Co(II). T. thermophilus CopA may efflux these metal ions. The metal-binding motif of most CsoR-family proteins contains C-H-C (Liu et al., 2007), but the corresponding residues of T. thermophilus CsoR are C-H-H. The Cu(II)-binding residues of Lentinus tigrinus blue laccase (Ferraroni et al., 2007), Carrella maxima laccase (PDB accession no. 3DIV) and Paracoccus pantotrophus pseudoazurin (PDB accession no. 3ERX) also contain two histidine and one cysteine residues. The crystal structure of T. thermophilus CsoR suggests that H5, a residue corresponding to which is not found in M. tuberculosis CsoR, is also involved in the binding of a metal ion. It may be that the metal ion-binding motif of T. thermophilus CsoR contains H-C-H-H, the same as that of E. coli RcnR, which binds Ni(II)/Co(II) and regulates expression of the nickel and cobalt efflux protein (Iwig et al., 2006, 2008). T. thermophilus CsoR may have properties of both CsoR and RcnR. B. subtilis CsoR, which may have an N-terminal histidine residue corresponding to that of T. thermophilus CsoR or E. coli RcnR (Fig. 1), can bind Ni(II), Co(II) and Zn(II), although these metal ions do not strongly regulate DNA binding in vitro (Ma et al., 2009a). Interestingly, the Cu(I)-binding affinity of the T. thermophilus CsoR_H70C mutant was lower than that of the wild-type, and Cu(I)-dependent negative regulation was not observed in vitro even with a 10-fold higher concentration of the ion than the protein, although the metal-binding motif of the mutant contains (H)-C-H-C, which is the same as that of the M. tuberculosis and B. subtilis CsoR proteins. Thus, H70, but not a cysteine residue, is necessary for T. thermophilus CsoR to act as a Cu(I)-sensing repressor. Since the DNA-binding affinity as well as the metal ion responsiveness differed between the wild-type and CsoR_H70C, H70 may be one of the critical residues that determines the structure and function of CsoR.

The mechanism of Cu(I)-dependent negative regulation of CsoR is unknown at the atomic level. X-ray crystal structure analysis showed that the C-terminal portion of the α2 helix of the DNA-unbound form of Cu(I)-bound CsoR (M. tuberculosis CsoR) bent toward the copper ion, but did not do so in the case of the DNA-bound apo-form of CsoR (T. thermophilus CsoR). This conformational change of the α2 helix might be a trigger for CsoR to be released from DNA. Further biochemical and structural
studies will elucidate the regulatory mechanism of CsoR, and the bacterial metal homeostasis system controlled via this transcriptional regulator.

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REFERENCES


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