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

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The *TTHA1719* gene from *Thermus thermophilus* HB8 encodes an orthologue of the copper-sensing transcriptional repressor CsoR. X-ray crystal structure analysis of *T. thermophilus* CsoR indicated that it forms a homotetramer. The structures of the CsoR monomer and dimer are similar to those of *Mycobacterium tuberculosis* CsoR. In the absence of copper ions, *T. thermophilus* CsoR bound to the promoter region of the copper-sensitive operon *copZ-csoR-copA*, which encodes the copper chaperone *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(II) and Cu(I) 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.

**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 1973, 251 and 14 ORFs, respectively (NCBI accession nos NC_006461–NC_006463). Structural and functional genomics studies have been carried out on this strain because most proteins from this organism are stable with respect to crystallization and functional analysis (Yokoyama et al., 2000). The intracellular copper ion level in this strain as
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 orthologue of CsoR. We also characterized the Thermotoga maritima 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). The 280 bp NdeI-BglII fragment of the plasmid was inserted under the control of the T7 promoter (NdeI-BamHI sites) of the *E. coli* expression vector pET-11a (Novagen). The 280 bp NdeI fragment using primers P01 and P02 (Supplementary Table S1), and then the Luria–Bertani (LB) broth containing 50 μg ampicillin ml⁻¹ for 16 h. The cells were resuspended in 60 ml of 20 mM Tris/HCl (pH 8.0) 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) 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 25,500 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 (NH₄)₂SO₄, and the bound protein was eluted with a linear gradient of 2.5–0 M (NH₄)₂SO₄. The target fractions were collected and then the expression vector pET-11a (Novagen) to construct pET11a-csoR. The second codon found in the chromosome, CCC, was converted to CCA in the expression vector. *E. coli* was added to the supernatant to a final concentration of 2.5 M; then, the supernatant was passed through a Ni-NTAagarose column (GE Healthcare) pre-equilibrated with 50 mM sodium phosphate buffer (pH 8.0) containing 0.15 M NaCl. The target fractions were collected and desalted by fractionation on a HiLoad 26/10 desalting 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 0.1 M sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl, and the proteins were diluted to 20.4 μM with a buffer containing 20 mM MES-NaOH (pH 7.0) and 0.2 M NaCl. Then, 5 μl CuCl₂ solution was added to 245 μl of the protein solution, followed by incubation at 55 or 25 °C for 6 min. Subsequently, 1 μl of 50 mM DTT or H₂O was added, and then after 5 min, the A₂₈₀ was measured.

**Crystallization.** Crystallization of Se-CsoR was performed by the siting-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.

**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 Rfree 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 diluted to 20.4 μM with a buffer containing 20 mM MES-NaOH (pH 7.0) and 0.2 M NaCl. Then, 5 μl CuCl₂ solution was added to 245 μl of the protein solution, followed by incubation at 55 or 25 °C for 6 min. Subsequently, 1 μl of 50 mM DTT or H₂O was added, and then after 5 min, the A₂₈₀ 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 2.0 μM with a buffer containing 20 mM MES-NaOH (pH 7.0) and 0.2 M NaCl. Then, 5 μl CuCl₂ solution was added to 245 μl of the protein solution, followed by incubation at 55 or 25 °C for 6 min. Subsequently, 1 μl of 50 mM DTT or H₂O was added, and then after 5 min, the A₂₈₀ was measured.

**In vitro transcription assay: preparation of templates.** The upstream region of the TTHA1718 gene (copZ) corresponding to
Table 1. X-ray data collection and refinement statistics

Values in parentheses are for the highest-resolution shell.

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<th>Statistic</th>
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<td>Unit cell parameters (Å, °)</td>
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<td>117 644</td>
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<td>No. of unique reflections</td>
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</tr>
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<td>5.6 (4.0)</td>
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<td>16.3 (4.0)</td>
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</tr>
<tr>
<td>Outliers (%)                         *</td>
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*R_{merge} = \sum_h \left| I_{h} - \langle I_h \rangle \right| / \sum_h I_h, where I_{h} is the \( h \)th measured diffraction intensity of reflection \( h \) and \( \langle I_h \rangle \) is the mean intensity of reflection \( h \).

†R_work = \sum_h \left| F_o - F_c \right| / \sum_h F_o, where \( F_o \) and \( F_c \) are the observed and calculated structure factors, respectively.

‡R_free is the R factor calculated using 10% of the data that were excluded from the refinement.

§Calculated with MolProbity.

positions 1 614 339–1 614 420 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 BamH–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 dNTP, 5.55 × 10⁸ Bq [z-³²P]dCTP (MP Biomedicals), 0.2 µM template DNA, 50 nM RNA polymerase-α² holoenzyme (RNAP) from *T. thermophilus* H88 purified as described previously (Vassylyeva 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 55 °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 × 10⁸ Bq [z-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 dNTP 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 × 10⁶ Bq [z-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 dyeoxy-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 1 614 339–1 614 420 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 BamH–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 dNTP, 5.55 × 10⁸ Bq [z-³²P]dCTP (MP Biomedicals), 0.2 µM template DNA, 50 nM RNA polymerase-α² holoenzyme (RNAP) from *T. thermophilus* H88 purified as described previously (Vassylyeva 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 55 °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 × 10⁸ Bq [z-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 dNTP 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 × 10⁶ Bq [z-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 dyeoxy-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
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 1 614 414–1 614 891 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, pHJK3 (Hoseki et al., 1999), and then the amplified fragments were ligated into a TA cloning vector, pGEM-T Easy (Promega). The plasmid carries 5′-TAATACGACTCACTATAGGG-3′ (HpaI and XhoI restriction sites in lower-case type) and 5′-ctgcaattattgatatcTATTgatatcTATTGTTG-3′ (PstI and EcoRV restriction sites in lower-case type) instead of the PstI site upstream of HTK and the PstI site downstream of HTK (Hoseki et al., 1999), respectively. The original PstI site on pGEM-T Easy was converted to 5′-CTCGCAT-3′ by site-directed mutagenesis 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). The HpaI–XhoI 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). The HpaI–XhoI 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-ΔcsoR. pGEM-ΔcsoR was transformed into the HB8 strain, and a kanamycin-resistant clone was isolated as a disruptant with respect to the csoR gene, as described previously (Hashimoto et al., 2001). We confirmed that the csoR gene was replaced by the HTK gene by genomic PCR with primers P13 and P14 (Supplementary Table S1).

**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 DUF156 superfamily like the *M. tuberculosis* and *B. subtilis* CsoR proteins, which show high 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 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).

**Construction 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.
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 Rwork and Rfree 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, E86 and 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 9.7 and 1.6 Å for chain A, and 8.9 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.](https://www.microbiologyresearch.org/article-pdf/10.1042/MB20070317.pdf)
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).
Copper ion-binding ability of *T. 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 *T. thermophilus* CsoR

According to the genome analysis results, *csoR* forms an operon with the *THHA1718* (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 that 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* transcription was in the palindromic sequence, suggesting 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 BLAcore analysis. A dsDNA containing the predicted binding site, 5’-GGCGTACCCACCCCCACCTGGGGTGAGGAG-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’-AGCCGAATTTCAGCTCGATGCTTGCTAGCAAATCGGGG-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 sensorgrams 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’-CCCACCCCCNNNNGGGGTGGG-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

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**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* PcpZ). 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.** 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. therophilus 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. therophilus 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. therophilus 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. therophilus 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. therophilus CsoR are C41, H66 and H70; that is, C65 of M. tuberculosis CsoR is not conserved in T. therophilus 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. therophilus 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. therophilus CsoR are C41, H66 and H70; that is, C65 of M. tuberculosis CsoR is not conserved in T. therophilus 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. therophilus CsoR, the CsoR H70C mutation was generated and tested for its ability to repress in vitro transcription in the presence of copper ions. The results showed that the H70C mutant had reduced activity compared to the wild-type CsoR, indicating that H70 plays a crucial role in copper ion binding.
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>
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<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>
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<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>
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<tr>
<td>TTHA1719 (csoR)</td>
<td>6.51 (0.01)</td>
<td>–</td>
<td>–</td>
<td>9.91 (0.00)</td>
<td>Copper-sensing transcriptional repressor</td>
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<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>
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<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>
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<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>
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<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>
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<td>TTHA1724</td>
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<td>1.77 (0.01)</td>
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<td>2.52 (0.00)</td>
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<td>TTHA1728</td>
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<td>3.67 (0.01)</td>
<td>2.08 (0.04)</td>
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<td>1.03 (0.26)</td>
<td>1.19 (0.14)</td>
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<td>1.19 (0.03)</td>
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<td>TTHA1732</td>
<td>6.89 (0.00)</td>
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<td>1.63 (0.01)</td>
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<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. 3b), 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. 6a). However, transcriptional derepression by Cu(I) was not observed even with a 10-fold higher concentration of Cu(I) than that of CsoR_H70C (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), Carrena 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 z2 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 z2 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.

ACKNOWLEDGEMENTS

We wish to thank Aimi Osaki for the protein purification, and Yuka Nonaka for the data collection at SPRing-8.

REFERENCES


Edited by: F. Sargent