Response to Copper Stress in *Streptomyces lividans* Extends beyond Genes under Direct Control of a Copper-sensitive Operon Repressor Protein (CsoR)*

Revised, March 17, 2012, Published, JBC Papers in Press, March 26, 2012, DOI 10.1074/jbc.M112.352740

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Background: Balancing copper homeostasis and bioavailability is critical for morphological development in *Streptomyces lividans*.

A copper-sensitive operon repressor protein (CsoR) has been identified in *Streptomyces lividans* (CsoR<sup>Sl</sup>) and found to regulate copper homeostasis with attomolar affinity for Cu(I). Solution studies reveal apo- and Cu<sup>I</sup>-CsoR<sup>Sl</sup> to be a tetramer assembly, and a 1.7-Å resolution crystal structure of apo-CsoR<sup>Sl</sup> reveals that a significant conformational change is necessary to enable Cu(I) binding. *In silico* prediction of the CsoR regulon was confirmed *in vitro* (EMSA) and *in vivo* (RNA-seq), which highlighted that next to the *csoR* gene itself, the regulon consists of two Cu(I) efflux systems involving a CopZ-like copper metallochaperone protein and a CopA P<sub>1</sub>-type ATPase. Although deletion of *csoR* has only minor effects on *S. lividans* development when grown under high copper concentrations, mutations of the Cu(I) ligands decrease tolerance to copper as a result of the Cu(I)-CsoR mutants failing to disengage from the DNA targets, thus inhibiting the derepression of the regulon. RNA-seq experiments carried out on samples incubated with exogenous copper and a ΔcsoR strain showed that the set of genes responding to copper stress is much wider than anticipated and largely extends beyond genes targeted by CsoR. This suggests more control levels are operating and directing other regulons in copper homeostasis beside the CsoR regulon.

* This work was supported by the University of Essex (to S. D., A. K. C., M. A. H., and J. A. R. W.), The Biochemical Society (to A. K. C.), and Leiden University and NWO (to E. V.).

** This article contains “Materials and Methods,” supplemental Figs. S1–S9, and Tables S1–S6.

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knock-out studies has suggested that secreted cuproproteins or cuproenzymes are needed for the development switch from vegetative to aerial mycelium to occur, but their identification and role in the development switch have not so far been elucidated (10, 11).

The copper proteome of Streptomyces coelicolor has been determined using bioinformatics approaches, revealing a rich assortment of putative extracellular cuproenzymes, redox proteins, and copper metallochaperone-like proteins (10, 12). An assortment of putative extracellular cuproenzymes, redox proteins involved in development, and copper metallochaperone-like proteins (10). An assortment of putative extracellular cuproenzymes, redox proteins involved in development, and copper metallochaperone-like proteins (10). An assortment of putative extracellular cuproenzymes, redox proteins involved in development, and copper metallochaperone-like proteins (10). An assortment of putative extracellular cuproenzymes, redox proteins involved in development, and copper metallochaperone-like proteins (10). An assortment of putative extracellular cuproenzymes, redox proteins involved in development, and copper metallochaperone-like proteins (10).

FIGURE 1. ClustalW2 multiple amino acid sequence alignment of copper-sensing CsoR proteins for which biochemical or structural data have been reported. Completely and partially conserved residues are boxed in dark and light gray, respectively. The conserved CufI-binding ligands are indicated (*) along with residues reported to play a role in the CufI-binding allosteric regulation of DNA (+). The UniprotKB accession numbers are Q8Y646 (L. monocytogenes), A6QIT1 (S. aureus), O32222 (B. subtilis), and P71543 (M. tuberculosis).

The founding member of the CsoR family was discovered in Streptomyces coelicolor (7). In S. coelicolor, the gene...
recorded by determining the dry biomass from 1.5-ml samples collected in pre-dried Eppendorf tubes, and mycelium was pelleted by centrifugation at 13,000 rpm for 10 min. After 12–16 h at 105 °C, the dry weight was determined using an analytical balance.

**Generation of a CsoR Deletion Mutant (ΔcsoR) of S. lividans 1326**—The 4136 gene encoding for CsoR Sl was deleted in S. lividans 1326 in a two-step process using the CRE-lox system (23). First, the gene (nt +4 to 399) was replaced by homologous recombination with an apramycin resistance cassette flanked by loxP sites. For this purpose, the upstream flanking region of SL4136 (−1370 to +3) and the downstream flanking region (+399 to 1680) were amplified from genomic DNA by PCR, including EcoRI, XbaI, XbaI, and HindIII sites, respectively, for cloning purposes. These two fragments and the apramycin resistance cassette flanked by loxP sites were cloned in the delivery vector pWHM3 that is unstable in S. lividans (23). Following protoplast transformation, recombinants that were apramycin-resistant but had lost the vector (thiostrepton resistance) were isolated. Second, an unstable plasmid encoding the Cre recombinase was introduced (23) and allowed for the excision of the apramycin resistance cassette on the lox sites. The resulting strain, ΔcsoR, has no coding sequence for CsoR Sl and has only a 61-nt “scar,” including two XbaI sites left in the genome. The ΔcsoR strain was analyzed by PCR to confirm the loss of 4136, the apramycin resistance cassette, and vector sequences.

**RNA Isolation and Transcriptome Analysis by RNA-seq**—Total RNA was isolated with Kirby mix according to standard procedures from mycelium in early log phase grown on NMMP supplemented with 0.5% glucose and mannitol (22). Cultures were induced with 400 μM Cu(II) for 2 h followed by total RNA isolation. RNA integrity was confirmed by agarose gel electrophoresis, and the absence of genomic DNA was checked by PCR. For the removal of ribosomal RNA, an Ambion kit was used. Samples were sent to BaseClear, an independent and accredited service laboratory for DNA-based research, and transcriptome analysis by RNA-seq was carried out. The sequences obtained on an Illumina sequencer were filtered for noncoding RNAs and analyzed with CLCbio bioinformatics software packages using the annotated S. coelicolor genome as reference. Expression values were expressed as reads/kb of exon model/million mapped reads (25), i.e. dividing the total number of exon reads (in this case one exon per reference sequence) by the number of mapped reads (in Millions) times the exon length (in this case the length of the reference sequence).

**Promoter Probing**—The DNA sequence (−300 to +3) upstream of SL4136 was obtained from genomic DNA by PCR introducing a flanking EcoRI and BamHI site to facilitate cloning in pIJ2585 digested with the same enzymes (26). The resulting plasmid, p4136-I, was introduced in strain M512 by protoplast transformation. Cultures were grown in triplicate in NMMP medium supplemented with 0.5% glucose, 0.5% mannitol, and 50 μg/ml apramycin. Strain M512 transformed with the empty vector was grown under the same conditions to obtain background readings. Following extraction of mycelium with methanol, the concentration of undecylprodigiosin was quantified from the absorbance at 530 nm and the extinction coefficient of 100,500 liters/mol·cm⁻¹ (27).

**Determination of Tyrosinase and Cytochrome c Oxidase Activity**—The wild type, S. lividans 1326, and the mutant strain ΔcsoR were each transformed with pLL703 (plasmid harboring the Streptomyces antibioticus melC operon under control of its own promoter). Four independent transformants of each strain were grown in liquid tryptic soy broth with 10% sucrose medium supplemented with 25 μM Cu(II). Tyrosinase activity was determined in spent medium with 10 μM 3,4-dihydroxy-L-phenylalanine in 100 mM phosphate buffer, pH 6.8, as substrate (28). Cytochrome c oxidase activity was visualized with N,N,N',N'-tetramethyl-p-phenylenediamine as substrate, essentially according to Refs. 29, 30.

**Cloning, Overexpression, and Purification of CsoR Sl from Escherichia coli**—The 4136 gene was cloned from S. lividans 1326 genomic DNA (supplemental “Materials and Methods”) and overexpressed in Escherichia coli using a pET28a (Kan’) vector (Novagen). This construct, designated pET4136, was transformed to E. coli BL21(DE3) cells, and single colonies were transferred to 2X YT medium (Melford) with kanamyacin (50 μg/liter) (Melford) at 37 °C. Overexpression of the N-terminal His-tagged CsoR Sl was induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (Melford) to a final concentration of 1 mM, and the temperature was decreased to 25 °C for overnight incubation. Cultures were harvested by centrifugation at 4000 rpm for 20 min at 4 °C, and the cell pellet was resuspended in 50 mM Tris/HCl, 500 mM NaCl (Fisher) and 20 mM imidazole (Sigma) at pH 7.5 (Buffer A). The resuspended cell suspension was lysed using an EmulsiFlex-C5 cell disrupter (Avestin) followed by centrifugation at 18,000 rpm for 20 min at 4 °C. The clarified supernatant was loaded onto a 5-ml nickel-nitrilotriacetic acid-Sepharose column (GE Healthcare) equilibrated with Buffer A and eluted by a linear imidazole gradient using Buffer B (Buffer A with 500 mM imidazole). A single peak at ~30% Buffer B was eluted from the column, and fractions were pooled and dialyzed overnight at 4 °C against 10 mM MES, pH 6.5, 150 mM NaCl, 2 mM diethiothreitol (DTT) (Melford), and 4 mM EDTA (Sigma) (Buffer C). Following dialysis, the N-terminal His tag was removed by incubating the protein at room temperature overnight with 125 units of thrombin (Sigma). The protein/thrombin mixture was reapplied to the nickel-nitrilotriacetic acid-Sepharose column (GE Healthcare), and the flow-through was collected and concentrated using a Centricon (VivaSpin) with a 5-kDa cutoff at 4 °C for application to a G-75 Sephadex column (GE Healthcare) equilibrated with Buffer C. Following dialysis, the N-terminal His tag was removed by incubating the protein at room temperature overnight with 125 units of thrombin (Sigma). The protein/thrombin mixture was reapplied to the nickel-nitrilotriacetic acid-Sepharose column (GE Healthcare), and the flow-through was collected and concentrated using a Centricon (VivaSpin) with a 5-kDa cutoff at 4 °C for application to a G-75 Sephadex column (GE Healthcare) equilibrated with Buffer C. Fractions eluting from the major peak of the G-75 column were analyzed by SDS-PAGE, and those deemed of good purity were concentrated and stored at −20 °C until required.

**Site-directed Mutagenesis of CsoR Sl and Cloning for in Vivo Studies**—The QuickChange site-directed mutagenesis method (Stratagene) was used to create the C75A and H100A mutants of CsoR Sl. Forward and reverse primers were designed with the respective nucleotide change(s) to create the desired mutation (supplemental “Materials and Methods”), and the pET4136 plasmid was used as template. The respective mutations were confirmed by DNA sequencing. For expression in S. lividans, the wild type and mutant CsoR Sl open reading frames (ORF)

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were cloned under control of the 4136 promoter and the constitutive ermE promoter. For this purpose, the 4136 promoter region was obtained by PCR from S. lividans 1326 genomic DNA introducing an NdeI site on the ATG start codon of the ORF, and the ermE promoter was obtained from plasmid pHM10a (31). The wild type, C75A, and the H100A ORFs were cloned downstream of the promoters in the low copy vector pHJ401 (32).

UV-visible and Circular Dichroism Spectroscopies—A Varian Cary 50 UV-visible spectrophotometer and an Applied Photopysics Chirascan circular dichroism (CD) spectrophotometer (Leatherhead, UK) both equipped with a thermostatic cell holder controlled with a Peltier system were routinely used. An extinction coefficient (ε) at 280 nm of 3105 M⁻¹ cm⁻¹ was calculated for the CsoR⁺ monomer. This value was used throughout to determine the concentration of apo-CsoR⁺ samples. Far-UV CD spectra at 20 °C with 20 μM CsoR⁺ in 10 mM potassium phosphate, 50 mM potassium fluoride, pH 7.0, were acquired in the range 260 to 190 nm.

Electrospray Ionization Mass Spectrometry (ESI-MS) and Atomic Absorption Spectroscopy (AAS)—CsoR⁺ samples for mass spectrometry were diluted 1:20 with a 50% methanol and 1% formic acid solution. Spectra were acquired on a Micromass Quattro Ultima triple quadrupole instrument using the following experimental parameters: capillary voltage 1.7 kV, cone voltage 80–120 V, and cone gas 100 liter/h. Acquisition and processing were carried out using MassLynx software (Waters, Manchester, UK). Metal content was determined with a Unicam 939/959 atomic absorption graphite furnace spectrometer.

Cu(I) Titrations and Determination of Binding Affinity—Apo-CsoR⁺ samples for experiments with Cu(I) were prepared in an anaerobic chamber (DW Scientific O₂, <2 ppm) by first incubating for 2–3 h with 2 mM DTT followed by desalting upon titrating in the Cu(I) solution. Competition assays were set up anaerobically with either BCA or bathocuproine disulfonate (BCS) (Sigma). Increasing protein concentrations (0–90 μM) were sealed in an anaerobic quartz cuvette (Hellma), and the absorbance change at 240 nm was monitored spectrophotometrically (JOURNAL OF BIOLOGICAL CHEMISTRY). Stoichiometry. The dissociation constant for Cu(I) ([Cu⁺]) was determined from competitive assays by assuming Reaction 1 (1),

\[
\text{apo-CsoR} + \text{CuL}_2 \leftrightarrow \text{Cu} - \text{CsoR} + 2L
\]

and by using Equation 1,

\[
K_d \beta_2 = \frac{([\text{apo-CsoR}]_0([\text{M-CsoR}]) - 1)}{([L]/[\text{ML}_2]) - 2)}[\text{ML}_2]
\] (Eq. 1)

where [L] is the total ligand concentration (BCA or BCS), and the overall formation constant (β₂) is 10^{17.2} M⁻² for [copper (BCA)_2]^{13⁻} and 10^{19.8} M⁻² for [copper (BCS)_2]^{13⁻} (34, 36). Assays were performed in duplicate, and the \( K_d \) value for a series was initially calculated for each individual solution and then averaged. By using the average \( K_d \) value, a simulated curve was plotted using Equation 1.

Bioinformatic Identification of CsoR⁺ Operator Targets—The computational prediction of CsoR⁺ cis-acting elements was performed as described previously (37). Experimentally validated binding sites of CsoR orthologues in B. subtilis (TAATACCTACGGGGGTATGG) (15, 38), S. aureus (ATATACCTATAGGGGTATAT) (17), Geobacillus thermodenitrificans (TTATACCGAAGGGGTATAT) (17), and M. tuberculosis (RicR.1, ATATACCCTACGGGGGTATAG; RicR.2, ATATACCTATAGGGGTATGG; RicR.3, ATATACCTACGGGTATCT; RicR.4, TTGTACCCCAGCGGGG) (39) were used to generate via the PREDetector program (40) a first weight matrix to identify similar putative cis-acting sequences in terms of (i) scores, (ii) interspecies conservation, and (iii) physiological meaning (hits related to copper sensitivity). Hits conserved among the selected four streptomycetes and identified upstream of copper utilization-related genes, i.e. upstream of copZ (copper chaperone) and upstream of the CsoRStrep orthologues, were used to generate a new weight matrix (named “CsoR streptomycetes”), more specific for predictions in Streptomyces species. Sequences used to generate the CsoR streptomycetes weight matrix were copZSCO, copZSCAB, copZSAV, and copZSAV, and csosCO, csosGR, csosSCAB, and csosSAV and are presented in supplemental Fig. S6, and reliable CsoR-binding sites identified in these four Streptomyces species are presented in supplemental Tables S2–S5. Putative CsoR-like sequences predicted in SCO were used to identify cis-acting elements in the closely related strain S. lividans.

Electrophoretic Mobility Shift Assays of CsoR⁺ Targets—Intergenic DNA fragments (208, 232, and 240 bp) containing the absorbance of the [CuL₂]^{13⁻} complex spectrophotometrically for L = BCA at 562 nm (ε = 7900 M⁻¹ cm⁻¹) and L = BCS at 483 nm (13,000 M⁻¹ cm⁻¹) (34, 35). By interpolating L, assays favoring competitive or noncompetitive Cu(I) binding could be set up, which for the latter led to an estimate of the binding stoichiometry. The dissociation constant for Cu(I) \( (K_d(Cu^+)) \) was determined from competitive assays by assuming Reaction 1 (1),

\[
\text{apo-CsoR} + \text{CuL}_2 \leftrightarrow \text{Cu} - \text{CsoR} + 2L
\]
predicted target sequence for CsoRSl identified by PREDetector were amplified from genomic S. lividans 1326 DNA as described in the supplemental “Materials and Methods”. DNA oligomers (Sigma) for use in EMSA studies were between 35 and 36 bp in length. Complementary pairs were annealed by heating at 96 °C in a water bath for 5 min and left to cool to room temperature overnight. 0.5 μM of a DNA oligomer target was incubated with concentrations of apo-CsoRSl monomer ranging between 4 and 30 μM in 10 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM DTT. Cu(I)-CsoRSl samples were either prepared by pre-loading apo-CsoRSl with a stoichiometric amount of Cu(I) in an anaerobic chamber before mixing with DNA samples or added directly to the DNA-protein complex under anaerobic conditions. All samples were incubated at room temperature for 30 min and then loaded (20 μl) to a pre-run 6% Tris/borate EDTA (TBE) polyacrylamide gel. Gels were stained for 30 min in an ethidium bromide solution followed by imaging.

**Crystallization and Structure Determination of Apo-CsoRSl**—Crystals of apo-CsoRSl were grown using the hanging drop vapor diffusion method at 20 °C. 1 μl of protein solution at a concentration of 15 mg/ml was mixed with an equal volume of reservoir solution containing 1.26 M ammonium sulfate, 0.1 M sodium citrate, pH 4. Crystals of dimensions ~0.2 × 0.2 × 0.2 mm grew within 1 week. A single crystal was transferred to a cryoprotectant solution containing 1.3 M ammonium sulfate, 0.1 M sodium citrate, pH 4. Crystals of dimensions ~0.2 × 0.2 × 0.2 mm grew within 1 week. A single crystal was transferred to a cryoprotectant solution containing 1.3 M ammonium sulfate, 0.1 M sodium citrate, pH 4, and 15% glycerol, prior to flash-freezing in liquid nitrogen. Crystallographic data were measured to 1.7 Å resolution at Diamond Light Source beamline I04 using an ADSC Q315r CCD detector and an x-ray wavelength of 0.9795 Å. Data were indexed using iMosfil (41) and scaled and merged using Scala (42) in the CCP4i suite. The structure was solved by molecular replacement in BALBES (43). The initial model was built into the electron density map using Buccaneer (44) and refined using Refmac5 (45). Riding hydrogen atoms were added when refinement of the protein atoms had converged. Models were rebuilt between refinement cycles in Coot (46), and the final structure was validated using the MolProbity server (47) and Coot. Coordinates and structure factors were deposited in the RCSB Protein Data Bank with accession number 4adz. A summary of data and refinement statistics and the quality indicators for the structure are given in Table 1.

### RESULTS

**Mass, Metal Content, Secondary Structure, and Assembly State of Purified CsoRSl**—Purified CsoRSl ran as a single band on an SDS-polyacrylamide gel (supplemental Fig. S1) and denaturing ESI-MS gave a single component spectrum with a species of molecular mass 14,739 Da, in excellent agreement with the predicted mass (supplemental Table S1). AAS revealed negligible copper or nickel ion content, and thus an apo-form of CsoRSl was purified. The isolated protein reduced 5,5′-dithiobis(2-nitrobenzoic acid) to give a protein/thiol ratio of 1:1.8, suggesting that the thiol groups of Cys-75 and Cys-104, putatively involved in Cu(I) binding, remain reduced under the purification conditions (see below). The CD spectrum of apo-CsoRSl is shown in Fig. 2A with the two negative minima at 222 and 208 nm being typical signatures for α-helical secondary structure. Using this CD spectrum, a secondary structure content of 51% α-helix, 8% β-strand, 13% loops, and 27% unordered was predicted using Dichroweb (48, 49). Size-exclusion chromatography using a calibrated G-75 column revealed a major peak eluting at a mass of ~74 kDa, suggesting that under native conditions apo-CsoRSl exists as a higher order assembly (Fig. 2B). This was confirmed from analytical ultracentrifugation (AUC) experiments (supplemental “Materials and Methods”) where sedimentation equilibrium scans were fitted to a single component model to give an average single species molecular mass of 59.3 kDa (supplemental Fig. S2A). Based on the mass determined using ESI-MS, the mass obtained from AUC is consistent with apo-CsoRSl existing as a tetrameric assembly (expected mass 58,955.6 Da).

**Apo-CsoRSl Binds Cu(I) with Attomolar Affinity**—An anaerobic titration of Cu(I) into apo-CsoRSl gave rise to absorbance changes in the UV region of the spectrum (Fig. 2C). The absorbance increase at 240 nm is indicative of the formation of Cys-thiolate copper coordination bonds, whereas the 320 nm changes in the UV region of the spectrum (Fig. 2C). This indicates that under the concentrations employed, BCA cannot compete with CsoRSl for Cu(I), and that 1 eq of copper is bound per CsoRSl monomer, corroborating the

| TABLE 1  |
|------------------|------------------|
| **Crystallographic data collection and processing statistics for apo-CsoRSl** |
| Values in parentheses refer to the outermost resolution shell (1.79 to 1.70 Å). |
| **Wavelength** | 0.9795 Å |
| **Resolution** | 45.9 to 1.70 Å |
| **Space group** | P2₁,2₁,2₁ |
| **Unit cell** | a = 41.63 Å, b = 54.55 Å, c = 91.75 Å |
| **Unique reflections** | 22,220 (2403) |
| **Completeness** | 93.8% (71.7%) |
| **Rmerge** | 0.058% (0.283%) |
| **Rcryst** | 12.1 (2.1) |
| **RE** | 0.185 |
| **ESU based on ML** | 0.229 |
| **Root mean square deviation bond lengths** | 0.017 Å |
| **Root mean square deviation bond angles** | 1.66° |
| **Ramachandran favored** | 100% |
| **Wilson B-factor** | 22.4 Å² |
| **Protein Data Bank accession code** | 4adz |
results from the UV titration and AAS experiments. Competition for Cu(I) between apo-CsoRS and [CuI(BCS)]3−/2H11002 was observed (Fig. 2D). Using Equation 1 and an apparent KD of 1019.8 M−1 for [CuI(BCS)]3−/2H11002 gave a KD(CuI) of 2.6 × 10−18 M. This KD(CuI) was used to simulate a fit of the data, as shown by the solid line in Fig. 2D. Duplicate data sets were obtained with varying [CuI]total or [BCS], and the average KD(CuI) for apo-CsoRS at pH 7.6 was 6.7 × 10−18 M. Competition between CsoRS and [CuI(BCS)]3− was also observed at pH 6.5, and an average KD(CuI) of 3.2 × 10−17 M was determined.

Growth and Development of S. lividans 1326 Versus That of ΔcsoR Strain—The affinity of CsoRS for Cu(I) was consistent with a role in sensing, buffering, and handling copper in the cytosol under all growth conditions, including stress elicited by elevated copper concentrations. To assess the effect CsoRS has on growth and morphological development of S. lividans 1326 under normal conditions and when copper stress is applied, a deletion strain (ΔcsoR) was constructed. Fig. 3 shows an example of growth on R5 agar media of the wild type S. lividans 1326 and the ΔcsoR mutant at increasing copper concentrations. At low concentrations of copper, no significant differences between the two strains were observed regarding vegetative growth, aerial growth, and sporulation (Fig. 3). This was also the case on various other solid media and in the presence of copper chelators (supplemental Fig. S4A). However, above 750 μM Cu(II), the ΔcsoR strain appears to be slightly more affected in morphological development and in the production of the red pigment.
pigment (undecylprodigiosin) than the wild type (Fig. 3). Copper therefore has a triphasic effect on development. Under conditions where all copper in the medium is bound by the chelator bathocuproine disulfonic acid, development is completely blocked (supplemental Fig. S4A). Stimulation of aerial hyphae production and spores occurs when the bioavailability of copper concentrations increase to 2–5 μM, and development is inhibited again at concentrations above 500 μM. This effect is not observed on MM or soy flower mannitol solid media, but on MM development is also severely retarded above 500 μM Cu(II) and inhibited completely at 1000 μM (supplemental Fig. S4B). Growth rate determinations in liquid defined media (NMMP) supplemented with Cu(II) in the range from 0 to 1000 μM corroborated the observations on the different solid media. Essentially no differences were seen between the growth rates of the wild type and the ΔcsoR strain under all conditions (Table 2 and supplemental Fig. S5).

Challenging the wild type and the ΔcsoR strain with stresses that could affect copper homeostasis, such as diamide (redox stress) and hydrogen peroxide, showed a similar response in both strains (data not shown), as is the case for the addition of the iron chelator bathophenanthroline disulfonic acid to the medium (supplemental Fig. S4A). However, when the in vivo maturation of two cuproenzymes was measured, a small but consistent difference between wild type and the ΔcsoR strain was observed. Both the activity of cytochrome c oxidase and the secreted heterologous tyrosinase (MelC2) are significantly higher in the ΔcsoR strain (Fig. 4). Because deletion of the csoR gene is not likely to affect the expression of the endogenous cox genes (as confirmed by the RNA-seq data, see supplemental Table S6A) or the heterologous melC operon, these data would suggest that maturation/incorporation of the copper cofactor is more efficient in the ΔcsoR strain.

X-ray Crystal Structure of Apo-CsoRS<sup>Δ</sup>—The crystal structure of apo-CsoRS<sup>Δ</sup> was determined to 1.7 Å resolution. Two protomers (chains A and B) were found in the crystallographic asymmetric unit of a crystal of CsoRS<sup>Δ</sup> with well defined electron density visible for residues 44–133 in each protomer. The overall protomer fold (residues 44–133) consists of three α-helices of varying lengths. No electron density was visible for residues 1–43 suggesting that these residues are disordered. By applying crystallographic symmetry, a tetramer assembly, consistent with AUC, was generated with chain A packing against chain C and chain B packing against chain D (Fig. 5A). A surface representation of the tetramer is shown in Fig. 5B indicating that CsoRS<sup>Δ</sup> has a “closed” tetrameric assembly as opposed to a donut-like structure reported for a CsoR-like protein from Thermus thermophilus and CsoR<sup>wt</sup> (7, 51). The overall fold of CsoRS<sup>Δ</sup> and the packing of the three α-helices of each protomer within the tetramer assembly are similar to that in the homodimer structure of Cu<sup>1</sup>-CsoR<sup>wt</sup> (Fig. 5C) (7). Superposition of the two structures by secondary structure matching gave a root mean square deviation in Cα positions of 1.98 Å. An extended α-helix 3 in CsoRS<sup>Δ</sup> appears to be the reason for the closed assembly such that in apo-CsoRS<sup>Δ</sup> the C termini of each pair of protomers pack together. Further differences between these structures are observed in the hairpin loop connecting α-helices 2 and 3, and at the N terminus of α-helix 1 (Fig. 5C). The largest differences are in the vicinity of the copper-binding sites, with residues 104–106 having deviations of >4 Å. The electrostatic potential of the CsoRS<sup>Δ</sup> tetramer reveals a central region of strong positive potential at the interface of two homodimers that extends out toward helix 1 (Fig. 5B). Negative potential is located at the start and end of helix-2 and flanks the central positive potential in the tetramer assembly (Fig. 5B).

From the sequence alignment in Fig. 1, Cys-75, His-100, and Cys-104 are predicted to be the copper ligands in CsoRS<sup>Δ</sup>. In the structure of the tetrameric assembly, His-100 and Cys-104 are located toward the end of helix 2 in each protomer, and Cys-75′ is located on a loop connecting α-helices 1 and 2 of an opposite protomer creating a putative intersubunit binding site, as found in CsoR<sup>wt</sup>. The side chain of Cys-75 has been modeled in two

| TABLE 2 |
| --- |
| Growth rates (doubling/h) of the wild type <i>S. lividans</i> 1326 and the ΔcsoR mutant in NMMP medium supplemented with the indicated [Cu(II)]. Standard deviation of the growth rates was 0.01. |
| | μ<sup>−1</sup> |
| | Strain | 0 | 100 | 400 | 750 | 1000 |
| | <i>S. lividans</i> 1326 | 0.27 | 0.28 | 0.27 | 0.27 | 0.27 |
| | ΔcsoR | 0.28 | 0.29 | 0.27 | 0.26 | 0.26 |

FIGURE 4. Effect of ΔcsoR on cytochrome c oxidase and tyrosinase activity in <i>S. lividans</i> 1326. A, 1000 spores were spotted on MM agar plates and incubated for 50 h at 30 °C. The <i>in vivo</i> activity of cytochrome c oxidase was determined using N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) by monitoring the production of the blue compound. The ΔcsoR mutant displays a quicker production of the blue compound than the wild type both on plates with and without added Cu(II). B, tyrosinase activity in the spent medium was determined with 3,4-dihydroxy-L-phenylalanine as substrate at the indicated time points and normalized for the biomass. The average tyrosinase activity of four transformants is plotted.
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FIGURE 5. Structure of apo-CsoRSl. A, physiologically relevant tetramer assembly, with protomers colored red (A), orange (B), blue (C), and cyan (D). The close packing of the C termini of all chains is apparent. The Cys residues predicted to be involved in copper binding are shown as sticks with the S atom colored yellow, B, electrostatic surface representation of the CsoRSl tetramer. Positive charges are indicated in blue and negative charges in red. C, superposition (calculated in the program Superpose, part of the CCP4 suite), based on secondary structure matching, of protomers A and C of the CsoRSl structure (blue) with the dimer of CsoRMtb (red). The copper-binding Cys residues from CsoRMtb and the corresponding residues in CsoRSl are shown as sticks. D, 2Fo − Fc electron density map, contoured at 1σ for the putative copper-binding region in the structure of CsoRSl. Hydrogen bonds are shown as dashed red lines. The proposed copper binding residues Cys-75 and Cys-104 lie some 7.6 Å apart and are separated by the imidazole side chain of His-100, which forms a hydrogen bond to a well-ordered water molecule. A sulfate anion is present at the protein surface, forming bonds to His-100 and His-103. E, comparison of the copper-binding region in CsoRSl and CsoRMtb, based on the superposition shown in C. A and B prepared using CCP4MG (S6) and C–E prepared using PyMOL.

slightly different conformations with occupancies of 0.7/0.3 (Fig. 5D), with the S atom pointing either toward or away from the His-100 side chain. It is apparent that, in the absence of a copper ion, the His-100 imidazolate in apo-CsoRSl intersects the two Cys ligands, which are some 7.6 (monomer A)/7.3 (monomer B) Å apart such that no disulfide bond between the two Cys residues is formed (Fig. 5D). The Ne2 atom of His-100 forms an H-bond (2.7/2.6 Å) with a well-ordered water molecule (Wat1), which further hydrogen bonds (2.7/2.8 Å) to the side chain Oε1 atom of Glu-122 in α-helix 3 (Fig. 5D). A further H-bond interaction (2.6/2.7 Å) involving the Oε1 atom of Glu-122 and the OH atom of Tyr-74 of an adjacent protomer is also observed (Fig. 5D). A number of sulfate ion-binding sites are present in the structure, consistent with the ammonium sulfate component of the crystallization solution. One of these sites is located in the vicinity of each of the putative copper-binding sites. This sulfate anion is clearly positioned to be within H-bond distance of the Nδ1 atom of His-100 (2.6/2.6 Å) and 2.9/3.1 Å from Nδ1 of His-103 (Fig. 5D). It is interesting to note that the position of the sulfate anion is close to that of the modeled copper ion in the 2.55 Å resolution CsoRMtb structure (7).

In Silico Identification of Putative CsoRSl Operator Targets—
The structural similarity of the 4136 gene product to Cuo-CsoRSl adds further credence to a role as repressor in S. lividans. Based on experimentally validated cis-acting sequences bound by B. subtilis, S. aureus, and M. tuberculosis CsoR orthologues (7, 15, 17, 38), we bioinformatically predicted putative CsoRSl operator sequences in S. lividans. Regulons tend to be highly conserved in distantly related species, and therefore to ensure the identification of highly reliable CsoRSl putative target genes, the computational prediction was performed in four Streptomyces species, S. coelicolor, S. scabies, S. avermitilis, and S. griseus (supplemental Tables S2–S5), so as to increase the reliability of cis-acting element predictions. Predictions from S. coelicolor were used to identify putative CsoR operator sequences in S. lividans. Three target sequences were selected from a stringent prediction procedure for further in vitro analyses. The highest score was 21.69, and the target sequence (AAATACCCCTGTTGGGTATAT) was located −42 nucleotides upstream from the start codon of csoR and −183 nucleotides upstream from the start codon of 4137 encoding a putative phosphate transport regulator (Fig. 6A). The identification of the most highly reliable operator sequence upstream of csoR itself strongly supports the validity of the prediction procedure and suggests autoregulation of CsoRSl expression as reported for CsoR orthologs in other bacteria (7, 16, 17). The other two targets had scores of 19.37 (TTATACCCCTAGGGTA-AGG) and 14.15 (GGGTACCCCTAGGGGTATAC) and were found to be located −25 nucleotides upstream of the gene 2730 and −83 nucleotides upstream of the gene 1045 (Fig. 6A). Both of these genes are part of a copZA-like operon, predicted to encode a CopZ-like copper metallochaperone protein and a CopA P1-type ATPase, which in tandem operate as a specific Cu(I) efflux system in many bacterial systems. Based on our computational predictions, the deduced consensus binding sequence of streptomyces CsoR orthologues corresponds to the 21-nt palindromic sequence ATATACCCCTNAGGGGTATAT, where positions 3–8 and 14–19 (underlined) appear to be the most conserved and thus probably the more crucial for CsoRSl recognition (supplemental Figs. S6 and S7).

EMSA Analysis of CsoRSl Operator Targets and Assembly State of DNA–CsoRSl Complex—To test whether the three operator sequences identified with PREDetector would bind CsoRSl in vitro, EMSAs were carried out. These were initially performed with intergenic regions consisting of 240 bp (1044/1045), 232 bp (2729/2730), and 208 bp (4136/4137) and with smaller 35/36-bp DNA oligomers, containing the CsoRSl operator sequence flanked by 10 or 11 bp (Fig. 6A). Incubation of the intergenic regions or oligomers with apo-CsoRSl resulted in the formation of a low mobility CsoRSl-DNA complex visualized by the retardation of the DNA in the EMSA (Fig. 6, B and C). No shift in mobility of a random DNA sequence was observed suggesting that binding of the targets to CsoRSl is specific (Fig. 6C). Incubation of the DNA oligomers with CuI-CsoRSl samples or anaerobic addition of Cu(I) to preincubated apo-CsoRSl-DNA complexes resulted in the absence of a band shift, and only the
high mobility band corresponding to the free DNA target was observed (Fig. 6C). This strongly suggests that once Cu(I) is bound to CsoRSl, the affinity for these small DNA fragments is significantly reduced such that a complex is not detected by EMSAs. For the intergenic regions, similar behavior is observed, but it is noted that some low mobility complex remains (Fig. 6B). DNA incubated with only Cu(I) was not affected in mobility (Fig. 6C). To determine the DNA:CsoRSl binding stoichiometry, size-exclusion chromatography was used (supplemental “Materials and Methods”). This revealed a 1:8 ratio (DNA:CsoR Sl monomer) or two tetramers of CsoRSl are required to bind the DNA operator (supplemental Fig. S8).

Effect of Mutating Residues Cys-75 and His-100 in CsoRSl—Size-exclusion chromatography and AUC measurements verified that the mutants do not disrupt the tetramer assembly state, and AAS indicated the absence of stoichiometric copper in the purified proteins. Both mutants showed an absorbance increase at 240 nm upon addition of Cu(I) under anaerobic conditions, saturating at ~1 mol eq of Cu(I) (Fig. 7A and supplemental Fig. S9), and for the H100A mutant, the absorbance increase at ~320 nm that was observed in the wild type protein upon addition of Cu(I) was absent (Fig. 7A). The $K_D$(Cu$^I$) values for the mutants were determined using BCA as a competitive probe (Fig. 7B). Duplicate data sets at pH 7.5 were acquired for both mutants with varying [Cu]$_{total}$ or [BCA], and the data averaged to give a $K_D$(Cu$^I$) of 1.3 $\times$ 10$^{-14}$ M and 9.8 $\times$ 10$^{-16}$ M for the C75A and H100A variants, respectively. Both mutants therefore display an increased $K_D$(Cu$^I$) compared with wild type CsoRSl.

The mutants were each able to bind to the three DNA operators as indicated by EMSA (Fig. 6D). Addition of Cu(I) to the mutant-DNA operator complexes did not result in the disappearance of the low mobility band or a substantial increase in the intensity of the high mobility (free) DNA band (Fig. 6D). This suggests that Cu(I) binding in the mutants does not alter the affinity for the DNA operator to the extent observed for the wild type and suggests that the allosteric mechanism of operator release or exposure is affected. This apparent inability of the CsoR mutants C75A and H100A to disengage in vitro from the DNA operator following Cu(I) binding raises the question of what the effect on growth is when these mutant proteins are expressed in the mycelium. The wild type and mutated CsoRSl genes were cloned on a low copy plasmid under control of their own promoter. These constructs did not show any effect on growth. Therefore, the CsoRSl wild type gene and the mutant genes encoding the C75A and H100A variants were cloned under control of a strong constitutive promoter, and the wild type and ΔcsoR strain were transformed with these constructs. Fig. 8 clearly shows that the mutant forms of CsoRSl have a strong inhibitory growth effect in both strains starting from 100 μM Cu(II). The effect of mutant CsoR expression is slightly
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FIGURE 7. H100A mutant binds Cu(I) with a reduced affinity. A, changes in the UV region of the apo-H100A mutant baseline spectrum upon addition of Cu(OCl) at pH 7.5 and 20 °C. The inset shows the increase in absorbance at 240 nm plotted as a function of [CuI]/[apo-H100A monomer]. B, determination of the Kd(CuI) for apo-H100A under copper-limiting conditions imposed by [CuI (BCA)]3 as a competitive probe. The inset shows the decrease in absorbance at 562 nm in the visible region of the spectrum upon increasing [apo-H100A], and Equation 1 was used to determine the Kd(CuI). The line represents a best fit to the data using Kd(CuI) 6.3 x 10^-11 M. Conditions used are as follows: 10–100 μM [apo-H100A], 54 μM [CuI]total, and 260 μM [BCA].

FIGURE 8. Effect of C75A and H100A CsoR Sl mutants on growth. S. lividans 1326 and the ΔcsoR mutant were transformed with pHJL401 (empty vector) or the same vector harboring the csoR gene (p4136) or the mutants C75A (pC75A) or H100A (pH100A) under control of the constitutive ermE promoter. Spores (1000) were spotted on MM agar plates containing varying [Cu(I)] and incubated at 30 °C. After 50–60 h of incubation, a confluent lawn was produced. Images were taken with a Leica M80 stereomicroscope equipped with a DFC295 camera. Note the higher toxicity of exogenous copper supply due to mutations preventing the copper-dependent modulation of CsoR Sl DNA binding ability.

more pronounced in the ΔcsoR mutant than in the wild type because the latter also produces CsoR from the genomic gene copy resulting in a mix of plasmid and genome encoded proteins. Upon prolonged incubation, the control transformants harboring the empty vector or the wild type CsoR Sl gene produce confluent growth with 250 and 500 μM Cu(II), although those expressing the mutant CsoRs do not. These observations support the in silico data indicating that CsoR Sl has control over the expression of genes that encode proteins involved in exporting excess copper ions and its own expression under copper stress conditions.

Mapping the Global Response of Wild Type S. lividans to Elevated Copper Levels and the ΔcsoR Gene by RNA-seq—RNA-seq is a second generation high resolution sequencing technique that enables gene expression profiling in an organism to be analyzed in response to a mutation or an external stimulus (52–54). We have applied RNA-seq to further our insight into the CsoR regulon in S. lividans by analysis of the transcriptome of liquid-grown cultures. The transcriptomes of wild type S. lividans strain 1326 grown without and with a 2-h exposure to 400 μM Cu(II) were compared with that of the ΔcsoR strain. From analysis of the RNA-seq data, both CopZ genes (gene numbers 2730 and 1045) and the 1044 gene encoding for an uncharacterized secreted protein are all induced by exogenous copper and in the ΔcsoR strain (Table 3). These data corroborate the in silico approach and identify these genes as bona fide CsoR Sl targets. The CopA-like ATPases (2731 and 1046) are also up-regulated under elevated copper conditions (Table 3) but less so in the ΔcsoR strain. Nevertheless, this suggests regulation by the same promoter as the cognate CopZ. It is noticeable that the RNA-seq data do not provide clear support for the copper induction of gene 4137 or the csoR gene, which was predicted from PREDetector analysis (Table 3 and supplemental Table S2). Therefore, the promoter of csoR was analyzed in a promoter-probing experiment. The data clearly show that transcription originating from the csoR promoter is both copper-inducible and CsoR-dependent (Fig. 9A). The low induction by a 2-h exposure to 400 μM Cu(II) seen in the RNA-seq analysis (1.3-fold, Table 3) is confirmed by the promoter probing experiment (Fig. 9A). It also shows that upon longer exposure to Cu(II) the transcription is induced to around 2-fold, a similar level as observed in the ΔcsoR strain.

Finally, the transcriptome response to either copper or deletion of the csoR gene is not limited to the genes reported in Table 3. The global response can be appreciated visually by the use of Venn diagrams (Fig. 9B). A large number of genes are up- and down-regulated in response to copper and deletion of the csoR, with a significantly greater proportion of genes being down-regulated in the ΔcsoR strain. A considerable overlap is present between copper induction and csoR deletion (Fig. 9B and supplemental Table S6, B–G). Together, these data indicate that the response to changes in copper homeostasis in S. lividans is much more extensive than only the direct CsoR Sl regulon reported in Table 3. Aside from the genes up-regulated in Table 3, there is no obvious enrichment of genes that encode proteins known to be directly related to copper homeostasis. However, a gene for a putative copper transporter (3964), part of an operon encoding for two putative copper chaperones, is down-regulated in copper-induced S. lividans but not in the csoR deletion (supplemental Table S6, E–G).

DISCUSSION

At the molecular level, our understanding of proteins involved in copper detoxification, storage, and trafficking has
Table 3: Expression levels obtained by RNA-seq of the genes predicted by PREDetector to be under control of CsoR_Sl along with other putative copper proteins up-regulated

| Gene          | Annotion                      | PREDetector score | S. lividans 1326 copper-induced (RPKM) | S. lividans 1326 copper-induced (RPKM) | ΔCsoR (RPKM) | ΔCsoR (RPKM) |
|---------------|-------------------------------|-------------------|----------------------------------------|----------------------------------------|--------------|--------------|
| CsoR          |                               | 4136              | 21.7                                   | 169.8                                  | 217.5        | 1.3          |
| Pit accessory protein |                       | 4137              | 21.7                                   | 418.2                                  | 470.5        | 1.1          |
| Membrane protein |                               | 3280              | 21.3                                   | 21.7                                  | 112.8        | 5.2          |
| CopZ          |                               | 2730              | 19.4                                   | 86.7                                   | 436.0        | 5.0          |
| Secreted protein |                               | 1044              | 14.2                                   | 4.0                                   | 17.1         | 4.2          |
| CopZ          |                               | 1045              | 14.2                                   | 0.0                                   | 9.8          | 0.0          |
| CopA-like P1-type ATPase |                       | 2731              | 32.4                                   | 65.5                                   | 2.0          | 25.5         |
| CopA-like P1-type ATPase |                       | 1046              | 3.4                                    | 9.2                                   | 2.7          | 4.8          |
| Uncharacterized protein with CusF domain |                       | 7265              | 3.2                                    | 23.0                                   | 7.3          | 18.0         |
| Uncharacterized protein with a putative type 1 copper site |                       | 6710              | 0.0                                    | 6.3                                   | 0.0          | 2.1          |

*The selected expression measure is the RPKM. It is defined as the reads/kb of exon model/Million mapped reads (25).

*Genes 2731 and 1046 are in the same operon as 2730 and 1045, respectively.

*Gene 7265 is the only putative copper-trafficking linked gene that is induced both by copper stress and by csoR deletion (see Table S6A for all putative copper proteins).

Figure 9: Induction of csoR by promoter probing and global differential gene expression. A, promoter activity in response to the addition of 400 µM Cu(II) in the wild type and the ΔCsoR strain is expressed as fold change relative to the wild type strain without addition of Cu(II). Note the copper-induced Red production in the S. lividans wild type background and the constant and copper-insensitive Red production in the S. lividans ΔcsoR background. B, Venn diagrams depicting a global overview of the up- and down-regulated genes (>2-fold reads/kb of exon model/million) upon Cu(II) induction of wild type S. lividans and in the ΔcsoR strain. The supplemental Table S6, A–G, reports the lists of genes affected.

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advanced considerably in recent times (1, 3, 4). The next challenge is to interface our molecular and mechanistic understanding of copper homeostasis with the global response of an organism to copper overload. In this study, we have extensively characterized a Cu(I)-CsoR metalloregulator from an organism to copper overload. In this study, we have extensively characterized a Cu(I)-CsoR metalloregulator from S. lividans and uncovered the direct and extended CsoR regulon.

Cu(I) Affinity for CsoR Is Unified but How DNA Is Engaged Remains Unresolved—Recent reports have highlighted the need for a unifying approach to accurately determine Cu(I) binding affinities in homeostatic cuproproteins (36, 50). The need for a unifying approach to accurately determine Cu(I) binding affinities in homeostatic cuproproteins (36, 50).

CsoR Sl has high specificity and affinity for operator DNA targets (1326 RPKM) compared to other characterized CsoR proteins in that it possesses an extended (43 amino acids) N-terminal tail (Fig. 1). Structural organization of this region was not revealed from the crystal structure as electron density was only observed from residue 44 onward. Likewise, the C-terminal extension in CsoR_Mtb (7) was not structurally observed, and in the absence of proteolytic cleavage, dynamics outside the “core” structure are likely to be relevant. The core structure of CsoR (SL) is very similar to that of Cu+-CsoRMtb (7) and has no recognizable DNA structural binding motif, such as a helix-turn-helix motif. Despite this, apo-CsoR (SL) has high specificity and affinity for operator DNA targets (Fig. 6). The absence of a recognizable DNA-binding motif leaves the question of how CsoR orthologues engage with their operator target unanswered. From our structure of CsoR_SL, we observe that the tetramer assembly creates a large continuous surface area with strong electrostatic charge centered in the middle of each face of the tetramer, which may be of signifi-
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Copper sensing in DNA-bound structures has been shown to be an important mechanism for regulation of copper homeostasis. In Streptomyces lividans, the copper-sensing repressor CsoR controls the expression of genes involved in copper uptake and efflux. EMSA studies have shown that CsoR can bind to DNA in the absence of copper ions, suggesting that allosteric regulation plays a role in copper sensing.

**Allosteric Regulation**

An hypothesis for the mechanism of allosteric regulation was proposed, based on the crystal structure of CuI-CsoRMtb. The structure revealed that CsoR must undergo a significant movement to complete the Cu(I) coordination sphere. It was suggested that the absence of a DNA-binding motif is the reason why two tetramers associate with the DNA, and that the allosteric coupling free energy was determined to be close to zero.

**CsoR Structure and Mutations**

Mutagenesis studies, using unnatural amino acid substitutions tested experimentally, showed that unnatural amino acids were designed to abolish the H-bonding capacity of the Ne2 atom of the imidazole ring so that, on binding Cu(I) to the N81 atom, a second coordination sphere H-bond network centered on the Ne2 atom could not form and trigger release of the DNA (Fig. 5E). Cu(I) binding to the non-native His-substituted CsoR structure did not significantly affect the DNA binding affinity, but the allosteric coupling free energy (ΔGₐ) was determined to be close to zero. This observation was taken to indicate that allosteric switching is initiated upon binding Cu(I) to the N81 atom of the His ligand, triggering the formation of a H-bond network to the Ne2 atom that results in dissociation from the operator DNA sequence. However, this H-bond network is also present in the apo-CsoRS wild-type structure and suggests that copper binding is an essential requirement for its formation. One difference in the network found in CsoRS is the presence of a bridging water molecule between His-100 and Glu-122 (Fig. 5D). In the Cu(I)-CsoRMtb structure, no such water molecule is observed, and the H-bond length between the Cu(I) coordinating His and the Glu is ~4 Å (7). This is indicative of a very weak interaction, which may lead to a destabilization of the DNA-bound structure. Whether or not this water molecule plays a role in the mechanism of allosteric Cu(I) regulation or in DNA binding is presently unclear. From Fig. 5E, it is clear that His-100 and Cys-75’ in the apo-CsoRS structure are closely aligned to the corresponding residues in the Cu(I)-CsoRMtb structure. However, Cys-104 in apo-CsoRS must undergo a significant movement to complete the Cu(I) coordination sphere (Fig. 5E). It is conceivable that the movement associated with the positioning of the Cys-104 side chain to enable Cu(I) coordination may be of significance in allosterically regulating the dissociation from the DNA operator.

**CsoR Regulator in Streptomyces**

A CsoR-responsive element prediction in four different Streptomyces species confirmed a very limited occurrence of highly conserved sequences that have been identified only upstream of one or two sets of orthologues of the copper chaperone copZ and the efflux ATPase copA, as well as upstream of the copper-sensing repressor csoR itself. EMSA studies revealed that CsoR recognizes specifically these sequences, and the effect of Cu(I) on DNA binding supports a mechanism of allosteric regulation most likely similar to other CsoR orthologues.

**RNA-seq analysis**

RNA-seq analysis corroborates the in silico predictions, revealing clear induction of transcripts for two operons encoding copper efflux systems (2730/2731 and 1045/1046) and the divergent expression of 1044. A secreted protein of unknown function but not predicted to bind copper. RNA-seq data also indicates that CsoR transcript levels, under normal conditions, are relatively high compared with the two copZ genes and suggest that a significant proportion of CsoR is constitutively present. The observation that expression of the mutant csoR genes (C75A and H100A) under control of their own promoter does not lead to growth inhibition at higher [copper] illustrates that CsoR controls its own transcription. Failure of the mutants to disengage from the DNA operator has been reported for other copper ligand variants of CsoR orthologues and appears to lead to permanent transcription repression, resulting in too low protein expression levels to block transcription of the other genes with CsoR-binding sites. The induction of copZ was not observed from the RNA-seq data (Table 3) but was confirmed after >2 h of incubation with exogenous copper (Fig. 9A). This suggests that the possibility that a modular system of CsoR repression may be in operation. Interestingly, the in silico analysis in S. griseus predicted four other CsoR-responsive elements suggesting a somewhat wider regulon in this strain (supplemental Table S5). The SGR3189 (cutC) gene encoding a putative copper homeostasis protein (cutC) is located upstream of a copA-like gene but is divergently transcribed. This offers the possibility that the CsoR-responsive elements could act for both copA and cutC in S. griseus. Two other CsoR-binding sites are found at position ~30 nt from the SGR5260 gene, which could encode for the first member of an operon, including a putative multi-copper oxidase (SGR5259) and at position ~36 nt from SGR1262 (cstR) encoding for a putative non-copper sensing member of the CsoR family.

**A Model of the Directly Regulated CsoR Regulon in S. lividans**

Our findings clearly show that CsoR directly acts to regulate a three-locus regulon. Under homeostasis conditions, all operator sequences are occupied by two tetramers of CsoR resulting in repression of transcription with some free apo-CsoR and CopZ (2730), and no further action is required. Medium [copper] will require a stronger response, achieved by derepressing the 2730 and 1044/1045 operons resulting in expression of CopZ and the copA P₁-type ATPases (Table 3). A further increase in [copper] will result in copper binding by the apo-CsoR still occupying the csoR operator. As a consequence, CsoR will be produced at a higher level and will assist in buffering copper in the cytoplasm. We assume that CsoR can also mediate the trafficking of copper by donating its copper to CopZ, which in turn will have the specificity to deliver the metal to its cognate CopA P₁-type ATPase for export. As soon as copper levels are restored, apo-CsoR begins to occupy the operator sequences, and the system returns to its “ground state.” This model assumes that the up-regulation of CsoR occurs later. Quite how this may be possible is not known, but promoter probing and RNA-seq data are consistent with this phenomenon.

What Can Be Learned About Copper Homeostasis in S. lividans from RNA-seq Analysis?

Although RNA-seq analysis clearly provides insight into the response of transcripts under the direct control of CsoR (Table 3), it is very evident that response to exogenous copper or the deletion of the csoR gene is a complex process (Fig. 9B and supplemental Table S6, 2017).
A–G). Over 400 genes show significantly higher expression in the ΔcsoR strain without presenting putative CsoRSI binding sequences in their adjacent genetic environments. Therefore, the expression of these genes must be responding in an indirect way to CsoRSI and may possibly be due to the copper -binding ability of CsoRSI rather than its DNA-binding ability. Indeed, the inactivation of csoR would somehow mimic copper overload due to the absence of free cytosolic apo-CsoRSI involved in buffering excess copper.

Of the genes annotated to encode cuproenzymes and putative copper transport proteins, the expression of some could be CsoRSI-dependent (supplemental Table S6A). For example, from the initial PREDetector analysis of the streptomycetes CsoR regulon, a fourth type of gene that could potentially be controlled by CsoRSI was identified (supplemental Table S2). Gene 3280 encodes for a membrane protein, but experimental validation of the predicted site was not included in our present EMSA studies due to the unusual location of the predicted cis-acting element at position +24 nt within the predicted 3280 coding sequence. Similar sequences identified at position +4 and −186 nt relative to the translational start of the 3280 orthologue in S. avermitilis and S. scabies are more appropriately located for controlling gene expression by a transcriptional repressor (supplemental Tables S3 and S4). The RNA-seq data highlighted a 5.2-fold higher expression of 3280 in RNA samples collected from copper -induced cultures, whereas the expression level in the ΔcsoR remains unchanged (Table 3). This suggests that 3280 is involved in copper homeostasis, and an as yet unidentified transcription control system plays a role when CsoRSI is no longer present due to the gene deletion. A closer inspection of the 3280 product predicts a protein (~200 amino acids) with four transmembrane helices and a putative Cys-Xaa-Xaa-Xaa-Cys-binding motif at the start of the predicted first transmembrane helix.

The 220 genes that are induced by both exogenous copper and ΔcsoR (Table S6D) indicate that the CsoRSI and copper overload regulons only partially overlap with each other. One of these overlap genes is 7265, annotated as a membrane protein with a CusF domain and therefore implemented in copper binding. RNA-seq data reveal this gene to have the greatest fold increase of all known and predicted genes that encode for copper -binding proteins/transporters (Table 3) and may therefore have an as yet uncharacterized role in copper homeostasis. Gene 6710 encoding a protein containing a putative type I copper site is not expressed in the control culture but does show a low level of expression in the csoR mutant and upon copper induction. However, it is not clear at present how this protein could be involved in copper homeostasis. The strongest transcription increase both in response to copper overload and in the ΔcsoR mutant, by 29- and 15-fold, respectively, is a putative [2Fe-2S] thioredoxin-like protein (SCOS830). A functional reason for this is unclear as is the case for the hundreds of other genes that are induced by either copper or by the ΔcsoR. It is reasonable to assume from these data that besides CsoRSI control, one or most likely several other control systems operate in copper homeostasis, e.g. redox homeostasis. One possibility may be that the way the copper excess regulon is stimulated requires more sophisticated and efficient mechanisms than the slow and energy-consuming means involving transcriptional control by a DNA-binding protein.

Acknowledgments—We thank Professor Michael Wilson for discussions on metal competition experiments; Katie Blundell for assistance and analysis of the AUC data. Christopher Beckwith is acknowledged for contributing to the initial stages of constructing site-directed variants of CsoRSI.

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Response to Copper Stress in *Streptomyces lividans* Extends beyond Genes under Direct Control of a Copper-sensitive Operon Repressor Protein (CsoR)

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*J. Biol. Chem.* 2012, 287:17833-17847.

doi: 10.1074/jbc.M112.352740 originally published online March 26, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M112.352740

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