

# Control of Copper Resistance and Inorganic Sulfur Metabolism by Paralogous Regulators in *Staphylococcus aureus*<sup>\*[5]</sup>

Received for publication, January 9, 2011, and in revised form, February 1, 2011. Published, JBC Papers in Press, February 21, 2011, DOI 10.1074/jbc.M111.220012

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All strains of *Staphylococcus aureus* encode a putative copper-sensitive operon repressor (CsoR) and one other CsoR-like protein of unknown function. We show here that NWMN\_1991 encodes a *bona fide* Cu(I)-inducible CsoR of a genetically unlinked *copA-copZ* copper resistance operon in *S. aureus* strain Newman. In contrast, an unannotated open reading frame found between NWMN\_0027 and NWMN\_0026 (denoted NWMN\_0026.5) encodes a CsoR-like regulator that represses expression of adjacent genes by binding specifically to a pair of canonical operator sites positioned in the NWMN\_0027–0026.5 intergenic region. Inspection of these regulated genes suggests a role in assimilation of inorganic sulfur from thiosulfate and vectorial sulfur transfer, and we designate NWMN\_0026.5 as CstR (CsoR-like sulfur transferase repressor). Expression analysis demonstrates that CsoR and CstR control their respective regulons in response to distinct stimuli with no overlap *in vivo*. Unlike CsoR, CstR does not form a stable complex with Cu(I); operator binding is instead inhibited by oxidation of the intersubunit cysteine pair to a mixture of disulfide and trisulfide linkages by a likely metabolite of thiosulfate assimilation, sulfite. CsoR is unreactive toward sulfite under the same conditions. We conclude that CsoR and CstR are paralogs in *S. aureus* that function in the same cytoplasm to control distinct physiological processes.

The Gram-positive opportunistic human pathogen *Staphylococcus aureus* is the causative agent of a wide range of hospital and community-acquired infections that are associated with

significant morbidity (1). With the incidence of methicillin-resistant strains increasing in previously low prevalence areas (2), new antibiotic therapies that target novel metabolic pathways are urgently needed. One approach is to target those processes that allow a pathogen to respond to environmental stresses that might change depending on the microenvironmental host niche in which the organism finds itself. Resistance to host-mediated copper killing of *Escherichia coli* (3), *Salmonella enterica* (4), and *Mycobacterium tuberculosis* (5, 6) and sulfur assimilation and cysteine biosynthesis in *M. tuberculosis* (7, 8) are two such processes. *S. aureus* is particularly sensitive to rapid killing when exposed to copper or copper alloy surfaces, justifying this therapeutic direction (9, 10).

*M. tuberculosis* CsoR<sup>6</sup> (copper-sensitive operon repressor) is a founding member of large family of regulators now known collectively to respond to Cu(I), Ni(II), and perhaps other stressors, the structural basis of which is not fully understood (11, 12). All CsoR family proteins lack a known canonical DNA binding domain and are projected to adopt the flat disc-shaped dimer of dimers homotetrameric structure characteristic of Cu(I)-sensing CsoRs, with individual dimers consisting of an antiparallel four-helix bundle flanked by a C-terminal  $\alpha 3$  helix (13, 14). Two cysteine residues on opposite subunits within a dimer make coordination bonds to the Cu(I) ion, with the third ligand a His from the  $\alpha 2$  helix (Cys<sup>36'</sup>, His<sup>61</sup>, Cys<sup>65</sup>), thus completing a trigonal S<sub>2</sub>N coordination complex (13). Additionally, two conserved second coordination shell residues, Tyr<sup>35'</sup> and Glu<sup>81</sup>, play critical roles in driving allosteric negative regulation of DNA binding by Cu(I) within the tetramer (15, 16).

Some bacteria encode more than one CsoR family member. For example, pathogenic mycobacterial species encode as many as five CsoR-like proteins (13) and all strains of *S. aureus* appear to encode at least two. Both *M. tuberculosis* and *S. aureus* also encode at least one CsoR-like protein that conserves only the two Cys that coordinate Cu(I) in CsoR but otherwise lacks all other requisite features of a Cu(I)-sensing CsoR (16). This subfamily CsoR-like protein is also found in other Gram-positive microorganisms, including *Bacillus subtilis* (YrkD) and *Streptococcus pneumoniae* (SPD\_0073), where their functions are

\* This work was supported, in whole or in part, by National Institutes of Health Grant R01 GM042569 (to D. P. G.), U54 AI057157 from the Southeastern Regional Center of Excellence for Emerging Infections and Biodefense (to E. P. S.), and R01 GM042025 (to R. A. S.).

[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental "Methods," Tables S1–S3, Figs. S1–S13, and "Results."

<sup>1</sup> Both authors contributed equally to this work.

<sup>2</sup> Supported by Grant T32 HL094296 to Vanderbilt University and a postdoctoral fellowship from the American Heart Association.

<sup>3</sup> Portions of this work were submitted to the Texas A&M University in partial fulfillment of the requirements for the Ph.D.

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<sup>6</sup> The abbreviations used are: CsoR, copper-sensitive operon repressor; MMTS, methylmethanethiosulfonate; EXAFS, extended x-ray absorption fine structure; ESI, electrospray ionization; OP, operator-promoter.

completely unknown. This wide distribution and involvement in multiple regulatory pathways highlights the importance of understanding the molecular processes governed by this family of proteins.

In this work, we characterize the regulation of two stress response pathways in *S. aureus* by paralogs of the CsoR family of DNA-binding proteins. These transcriptional regulators are the copper sensor CsoR and a novel regulator denoted CstR (CsoR-like sulfur transferase regulator), which respond to distinct stressors with no detectable regulatory cross-talk in the cell.

## EXPERIMENTAL PROCEDURES

**Construction of  $\Delta$ csoR and  $\Delta$ cstR Deletion Strains**—The  $\Delta$ csoR mutant was constructed using established methods (17). Briefly, a PCR amplicon beginning 45 bp upstream of the corrected putative *csoR* (NWMN\_1991) ORF containing the ~1000 bp upstream sequence was amplified using primers CCCGGGAAAACACAACGTC AACACAAAG and GGGGCAAGTTTGTACAAAAAAGCAGGCTTTTACCTAAGTATCATCACC. Another amplicon containing ~50 bp of the putative *csoR* ORF together with ~1000 bp downstream of the putative *csoR* ORF was amplified using primers CCCGGG-CAGGAAGAGGCAATGGAAG and GGGGACCACTTTGTACAAGAAAGCTGGGTCTTTATCGTTGGTTTCGTCAC. These PCR-generated fragments were ligated together and cloned into pCR2.1 (Invitrogen). Next, the combined fragments were amplified using primers specific for 5' and 3' flanking sequences and the resultant PCR product was recombined into pKOR1 and used for allelic replacement into *S. aureus* strain Newman as described (17). An exactly analogous strategy was used to create the  $\Delta$ cstR deletion strain with the exception that primers GGGGACAAGTTTGTACAAAAAAGCAGGCTTTTCTTTTTTCATTACGTAGCGC and CCCGGGGTCATACCTCCACTTTTAATTG, and CCCGGGATTGGTGAAAAGTAAGTAATGG and GGGGACCACTTTGTACAAGAAAGCTGGGTACGTAATTTTAATAGCTTCG, were used to amplify the 5' and 3' fragments, respectively.

**Quantitative PCR**—To prepare samples for RNA extraction, 5-ml cultures were grown overnight in 15-ml conical tubes at 37 °C in supplemented Chelex-treated RPMI (NRPMI) for *copA* expression experiments or TAB for NWMN\_0026-NWMN\_0029 expression experiments. The next morning the cultures were back diluted 1/100 into 5 ml of NRPMI in a 15-ml conical tube with or without 1 mM MnCl<sub>2</sub> or 1 mM CuSO<sub>4</sub> or TSB. The cultures were grown for 4 h at 37 °C with shaking at 180 rpm. At the end of the incubation, an equal volume of 1:1 acetone/ethanol was added to the cultures and the samples were frozen at -80 °C. To harvest RNA, the samples were thawed on ice and centrifuged to pellet the bacteria. The supernatant was removed and the bacterial pellet was air dried. RNA was harvested as previously described using a combination of Tri-Reagent (Sigma) and RNeasy Minikit purification (Qiagen, Valencia, CA) (18) with the exception that after the addition of TRIzol the samples were transferred to bead beater tubes and processed at 6 m/s for 40 s in a bead beater to aid in cell lysis. Random hexamers and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) were used to generate

cDNA. Quantitative PCR was performed using the iQ SYBR Green Supermix (Bio-Rad) and the primer pairs as indicated under [supplemental Table S3](#) (18). Quantitation of 16 S ribosomal RNA was used to normalize each sample.

**Bacterial Expression Plasmid Construction and CsoR Purification**—The complete open reading frame annotated as locus tag NWMN\_1991 in *S. aureus* strain Newman (nucleotides 2212576–2212914; locus AP009351) (19) was PCR amplified and subcloned into pET3d between the NcoI and BamHI restriction sites. The resultant recombinant protein showed very poor solubility and no DNA binding to a 39-bp DNA derived from the promoter region of the *S. aureus copA* gene (see Fig. 1A) (data not shown). Further inspection of the DNA sequence of NWMN\_1991 revealed a second initiation codon positioned at nucleotide 2212869, resulting in ORF 15 codons shorter than that annotated as NWMN\_1991 and a consensus ribosome binding site just upstream of this initiation codon. A multiple sequence alignment of *bona fide* Cu(I)-sensing CsoRs revealed that no others contained an extended N-terminal region ([supplemental Fig. S1](#)). Therefore, the region corresponding to nucleotides 2212576–2212869 was hypothesized to represent the actual ORF corresponding to locus tag NWMN\_1991 and was therefore PCR amplified from genomic DNA and subcloned into pET3d between the NcoI and BamHI restriction sites. The second residue was changed to an alanine as a result (T2A) of the subcloning and is referred to as wild-type CsoR here. Amino acid substitutions were introduced into this expression plasmid by site-directed QuikChange mutagenesis, and the sequences of all resultant plasmids were verified by DNA sequencing.

Biochemical experiments confirmed the designation of the protein encoded by NWMN\_1991 as CsoR and is therefore referred to as such (see below). Plasmids carrying wild-type or mutant *Sau* CsoRs were transformed into *E. coli* BL21(DE3)/pLysS to ampicillin resistance. A single colony from an LB agar plate containing 100 mg/liter of ampicillin was inoculated into 200 ml of LB medium containing 100 mg/liter of ampicillin and grown overnight in a 37 °C shaker. 20 ml of the overnight culture was then used to inoculate 1 liter of the same LB medium and grown at 37 °C until  $A_{600}$  reached 0.6–0.8. 0.4 mM Isopropyl 1-thio- $\beta$ -D-galactopyranoside was then added and cells were grown for an additional 2 h prior to harvesting by low speed centrifugation. Cells were resuspended in 200 ml of Buffer E (25 mM Hepes, pH 7.0, 2 mM EDTA, 2 mM DTT) and lysed by sonication. After low speed centrifugation, CsoR was largely found in the lysis pellet, but was readily recovered in supernatant by stirring at 4 °C overnight in the same lysis buffer. 0.15% (v/v) of polyethyleneimine was added to the supernatant to precipitate the nucleic acids. Both wild-type and C41A *Sau* CsoRs were found in the polyethyleneimine pellet, which was then resuspended in Buffer E containing 0.5 M NaCl and reprecipitated, with the supernatant containing CsoR. In contrast, H66A *Sau* CsoR was found principally in the polyethyleneimine supernatant fraction. Each supernatant containing *Sau* CsoR was then subjected to ammonium sulfate precipitation and the resulting pellet resuspended in Buffer E and dialyzed exhaustively against Buffer E containing 0.05 M NaCl. The sample was then purified on a Q Fast Flow column with Buffer

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E using a salt gradient of 0.05–0.5 M NaCl. Fractions containing *Sau* CsoR were combined and concentrated to a final volume of ~3 ml. 1 ml of the resultant protein was then loaded onto a Superdex 200 30/100GL size exclusion column (GE Healthcare) pre-equilibrated with Buffer E containing 0.3 M NaCl. The fractions containing *Sau* CsoR were combined and dialyzed against Buffer E containing 0.05 M NaCl and loaded onto a MonoQ column for further purification. Fractions from the MonoQ column containing *Sau* CsoR were then pooled and concentrated to a volume of ~6 ml and dialyzed into Buffer S (10 mM Hepes, 0.2 M NaCl, pH 7.0) in an anaerobic glovebox. The purity of the final CsoRs was estimated by visualization of Coomassie-stained 18% Tris glycine SDS-PAGE gels to be  $\geq 90\%$  in each case. Protein concentration was determined by using a  $\epsilon_{280} = 1615 \text{ M}^{-1} \text{ cm}^{-1}$ . The free thiol content was determined by the 5,5'-dithiobis(nitrobenzoic acid) assay to be more than 90% of expected value in each case (2.0 expected) (13, 20). Less than 0.1% copper was detected by flame atomic absorption spectroscopy in all purified protein samples carried out as previously described (21).

**CstR Purification**—CstR is encoded by the complementary strand of nucleotides 37974–38234 in the *S. aureus* strain Newman genome. CstR was expressed in BL21(DE3)/pLysS cells under control of the *lac* repressor with coding sequences PCR-amplified and subcloned into pET3a between the NdeI and BamHI sites. Protein expression was induced with 1 mM isopropyl 1-thio- $\beta$ -D-galactopyranoside when the cultures had reached an optical density ( $A_{600}$ )  $\approx 0.6$  and allowed to grow for an additional 4–5 h at 37 °C at which time the cells were pelleted by centrifugation and stored at –20 °C overnight. The cell pellet was resuspended in 50 mM Hepes, 4 mM DTT, and 5 mM EDTA at pH 7.0 (Buffer A) with 1 M NaCl added to enhance the solubility of CstR. The solution was clarified by centrifugation. Standard 0.2% (v/v) polyethyleneimine precipitation removed most nucleotide contamination and the protein was then precipitated with 500 g/liter of  $(\text{NH}_4)_2\text{SO}_4$ . The ammonium sulfate pellet was resuspended in Buffer A and extensively dialyzed against Buffer A plus 50 mM NaCl at 4 °C resulting in CstR precipitation. The dialysate was clarified by centrifugation and the pellet resuspended in degassed Buffer A with 1 M NaCl. Gel filtration chromatography using Superdex-200 in extensively degassed Buffer A (1 M NaCl) yielded pure CstR (>95%) as visualized on an 18% acrylamide gel. A final anion exchange chromatography step was necessary to remove residual nucleotide contamination; in Buffer A (degassed), nucleotide-free CstR is present in the flow through at 300 mM NaCl. Dialysis into experimental buffer was carried out under an inert atmosphere (Vacuum Atmospheres glovebox). CstR was stored anaerobically at –80 °C.

**Cu(I) X-ray Absorption Spectroscopy**—Wild-type *Sau* CsoR was mixed with 0.8 mol eq of Cu(I) in 10 mM Hepes, 0.2 M NaCl, 30% (v/v) glycerol, pH 7.0, in an anaerobic environment and concentrated to a final protein concentration of  $\approx 0.5$ –1.0 mM. Samples were loaded into standard XAS cuvettes or 5-well polycarbonate XAS cuvettes and immediately frozen in liquid  $\text{N}_2$ . XAS data were collected at Stanford Synchrotron Radiation Lightsource (SSRL) on beamline 9-3. Extended x-ray absorption fine structure (EXAFS) data analysis was performed using

EXAFSPAK software, using *ab initio* phase and amplitude functions computed with FEFF version 7.2, according to standard procedures as described (15, 16, 21).

**Cysteine Modifications and Analysis by Mass Spectrometry**—100  $\mu\text{M}$  of unmodified and fully reduced CsoR or CstR (20  $\mu\text{M}$  protomer) were incubated with various concentrations of sodium thiosulfate ( $\text{Na}_2\text{S}_2\text{O}_3$ ), sodium sulfide ( $\text{Na}_2\text{S}$ ), methylmethanethiosulfonate (MMTS), or sodium sulfite ( $\text{Na}_2\text{SO}_3$ ) in 25 mM Hepes, pH 7.0, 0.2 M NaCl, at 25 °C for 17 h. All chemicals were reagent grade quality and obtained from Sigma or AlfaAesar. Quantitation of reaction products obtained with intact proteins was carried out by LC-ESI-MS on an Agilent 1200 HPLC-6130 MSD Quadrupole instrument fitted with a C18 column using a 5–95% acetonitrile gradient in 0.1% formic acid. These data were processed with ProTrawler (BioAnalyte Software). To determine the nature of the cross-linked peptide in sulfite-treated CstR, 1 unit of proteomics grade trypsin, resuspended in degassed water, was incubated with 100  $\mu\text{M}$  of 20  $\mu\text{M}$  apo-CstR or 20  $\mu\text{M}$   $\text{Na}_2\text{SO}_3$ -treated CstR under rigorously anaerobic conditions overnight in 25 mM Hepes, pH 7.0, 0.2 M NaCl, at 25 °C, and ESI-MS data were recorded and analyzed in the same way.

**Fluorescence Anisotropy Titrations**—Fluorescein-labeled dsDNA constructs were prepared as previously described (16) and diluted to 5 or 10 nM as indicated under strictly anaerobic conditions. 1–5- $\mu\text{l}$  aliquots of protein were anaerobically injected into 2 ml of dsDNA and allowed to equilibrate for 3–5 min. Fluorescence anisotropy,  $r_p$ , was measured with an ISS PC1 spectrofluorometer using  $\lambda_{\text{ex}}$  of 495 nm. The signal was normalized to starting ( $r_o$ ) and ending ( $r_{\text{complex}}$ ) anisotropies, the data were plotted *versus* total protein concentration (monomer) and fit to a two nondissociable tetramer binding model using DynaFit (22). The binding of CstR to unlabeled OP1 variants was conducted using a standard competition assay (23).

## RESULTS

**CopA Expression Is Induced by Copper Salts in a Manner That Requires the Gene Encoded by NWMN\_1991**—*S. aureus* strain Newman as well as all other sequenced staphylococci contain a homolog (NWMN\_1991) of the Cu(I) sensor CsoR from *M. tuberculosis* (*Mtb*) (13). This homolog conserves all previously identified critical features of the Cu(I)-dependent metalloregulatory switch (16) (supplemental Fig. S1). We therefore designated NWMN\_1991 as CsoR and predicted that that it would regulate the transcription of *copA*, a proposed copper exporter, in a Cu(I)-dependent manner (Fig. 1A). To test this, we constructed a  $\Delta\text{csoR}$  derivative and monitored the expression of *copA* by quantitative RT-PCR in wild-type (WT) and  $\Delta\text{csoR}$  in response to 1 mM  $\text{CuCl}_2$  or  $\text{MnCl}_2$  (Fig. 1B). Consistent with previous reports, *copA* expression is induced in the presence of copper salts by  $\approx 6$ -fold (24, 25), but not by Mn(II) (Fig. 1B). The  $\Delta\text{csoR}$  strain has elevated *copA* expression that is nearly independent of copper (Fig. 1B) suggesting that CsoR is responsible for controlling the copper stress response in *S. aureus*. Consistent with this finding is the observation that  $\Delta\text{csoR}$  is detectably more resistant to copper toxicity *versus* the wild-type strain (supplemental Fig. S2).

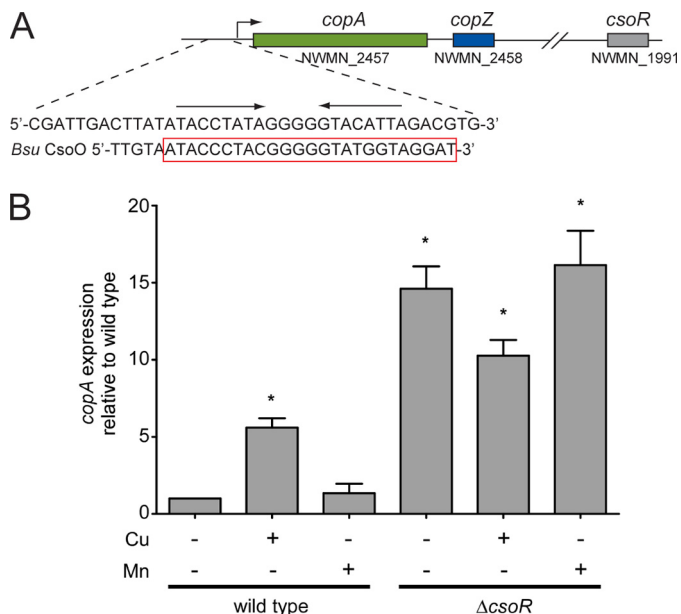


FIGURE 1. A, genomic location of *copA*, *copZ*, and the predicted *csoR* in *S. aureus* Newman. The DNA sequence in the *copA* promoter region is also shown compared with the *Bsu copZA* operator, which has been shown to be regulated and bound tightly by *Bsu* CsoR (15, 20). B, analysis of *copA* transcription by RT-PCR in WT and  $\Delta$ *csoR* grown in the absence or presence of 1.0 mM  $\text{CuCl}_2$  (Cu) or  $\text{MnCl}_2$  (Mn) as indicated. The results are the average of four independent experiments. \*,  $p < 0.05$  determined by Student's *t* test. Error bars = S.D.

*Sau* CsoR Binds 1 mol eq of Cu(I) per Monomer with High Affinity and Adopts a Three-coordinate  $S_2N$  Complex—A direct anaerobic titration of  $\text{CuCl}$  into apo *Sau* CsoR results in intense ligand to metal charge transfer absorption at  $\approx 240$  nm with an  $\epsilon \approx 15,000 \text{ M}^{-1} \text{ cm}^{-1}$  (supplemental Fig. S3) and a 1:1 Cu(I):protomer stoichiometry (supplemental Fig. S3, inset). Similar features characterize Cu(I) binding to *Mtb* and *Bsu* CsoRs (13, 15, 16). The Cu(I) binding affinity ( $K_{\text{Cu}}$ ) was further quantified using an anaerobic bathocuprione disulfonate competition assay with  $\log K_{\text{Cu}}$  determined to be  $18.1 \pm 0.5$  (supplemental Table S1). Substitution of Cys<sup>41</sup> or His<sup>66</sup> (equivalent to essential residues Cys<sup>36</sup> and His<sup>61</sup> in *Mtb* CsoR) (13) with alanine results in a significant decrease in  $K_{\text{Cu}}$  with  $\log K_{\text{Cu}}$  of  $14.5 \pm 0.1$  and  $15.3 \pm 0.1$  for C41A and H66A CsoRs, respectively (supplemental Table S1).

The Cu(I) coordination geometry was further investigated by x-ray absorption spectroscopy. As shown in Fig. 2A, the pre-edge peak of Cu(I)-CsoR at 8940 eV is consistent with a  $1s \rightarrow 4p$  excitation typical for 3-coordinate Cu(I) (13, 15, 26). The copper K-edge EXAFS spectrum as well as its Fourier transform for *Sau* CsoR with the best fit are shown in Fig. 2, B and C, respectively. The fitted parameters are compiled in supplemental Table S2. The fit suggests two Cu-S interactions at 2.20 Å and one Cu-N/O interaction at 2.01 Å. The significant outer shell scattering at 3–4 Å suggests that the third ligand is a histidine residue. *Sau* CsoR His<sup>66</sup> corresponds to His<sup>61</sup> in *Mtb* CsoR and His<sup>70</sup> in *Bsu* CsoR (13, 15) and the Cu(I) binding affinity is significantly decreased in the H66A mutant (supplemental Table S1). These data reveal that His<sup>66</sup> is a Cu(I) ligand that together with Cys<sup>41</sup> and Cys<sup>70</sup> complete the  $S_2N$  coordination complex. DNA binding experiments (supplemental Fig. S4)

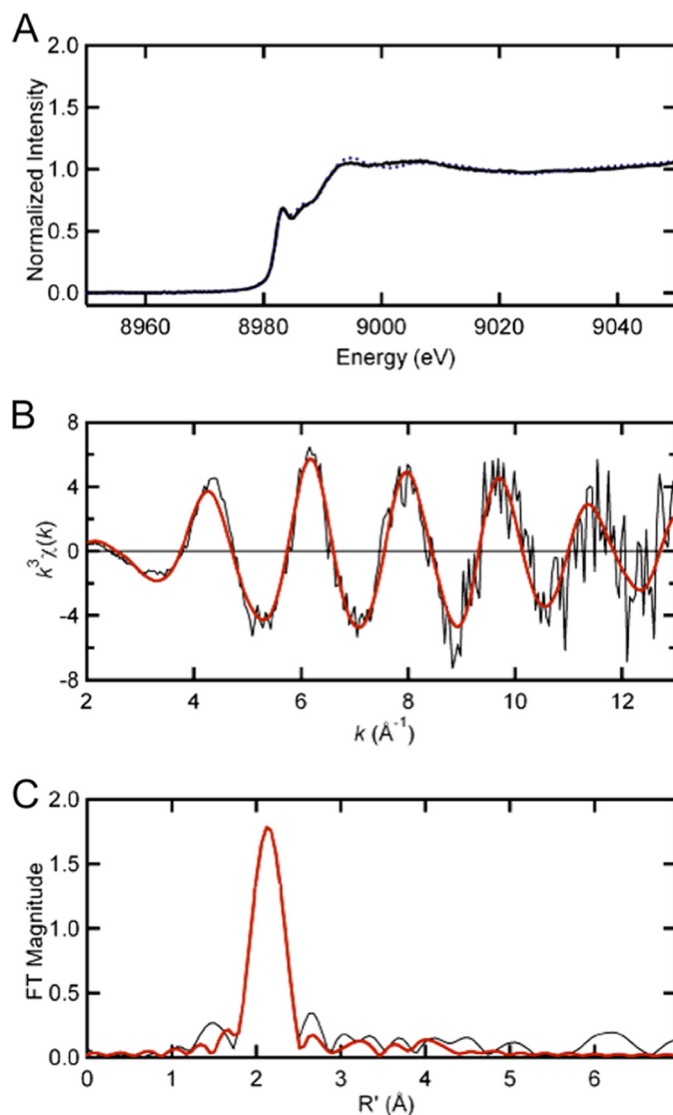
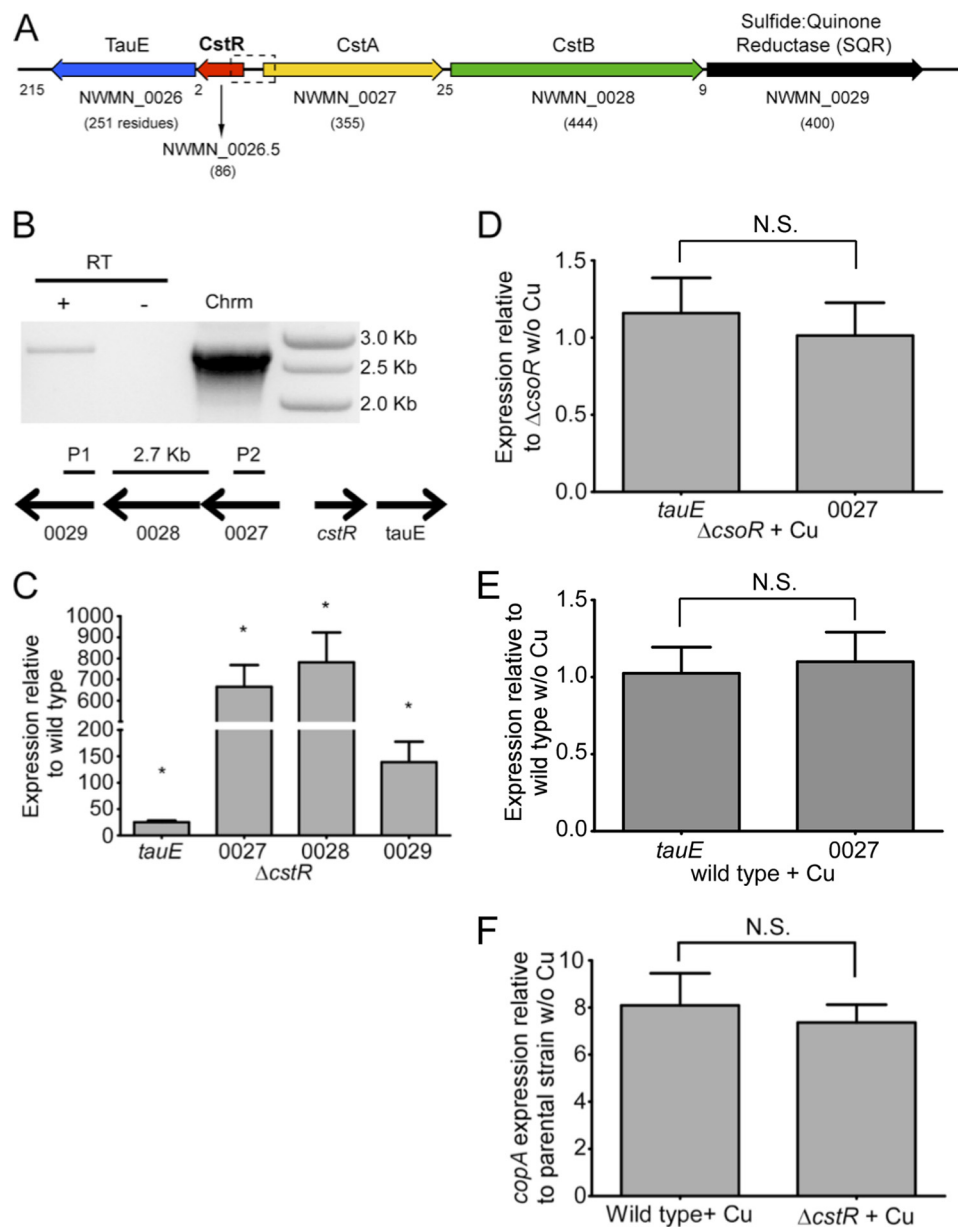


FIGURE 2. X-ray absorption spectroscopy (XAS) of Cu(I)-bound *Sau* CsoR. A, copper K-edge x-ray absorption edge spectra of Cu(I)-bound WT *Sau* CsoR (solid black) and *Mtb* CsoR 1–106 (dashed blue) (16). The copper K-edge EXAFS spectrum and Fourier transforms ( $k^3$  weighted,  $k = 2\text{--}13 \text{ \AA}^{-1}$ ) for Cu(I)-bound WT *Sau* CsoR are shown in panels B and C, respectively. The solid red curves in panels B and C represent the best fits with parameters compiled under supplemental Table S2.

show that Cu(I) binding to this site inhibits *cop* operator binding, whereas alanine substitutions of each of two Cu(I) ligands, Cys<sup>41</sup> and His<sup>66</sup>, alters the DNA binding properties of *S. aureus* CsoR significantly (13, 16).

*CstR* (NWMN\_0026.5) Is a Repressor of an Operon Predicted to Function in Sulfur Metabolism—The experiments described above reveal that locus tag NWMN\_1991 in *S. aureus* strain Newman encodes a *bona fide* Cu(I)-regulated CsoR. It was next of interest to determine the function of a previously unannotated open reading frame positioned between the NWMN\_0026 and NWMN\_0027 genes (Fig. 3A). This open reading frame, which we designate locus tag NWMN\_0026.5, encodes a protein that is 35% identical and 65% similar to CsoR and is annotated in every other available *S. aureus* genome (supplemental Fig. S5). An analysis of the deduced amino acid sequence of NWMN\_0026.5 reveals a CsoR/RcnR “WXYZ”

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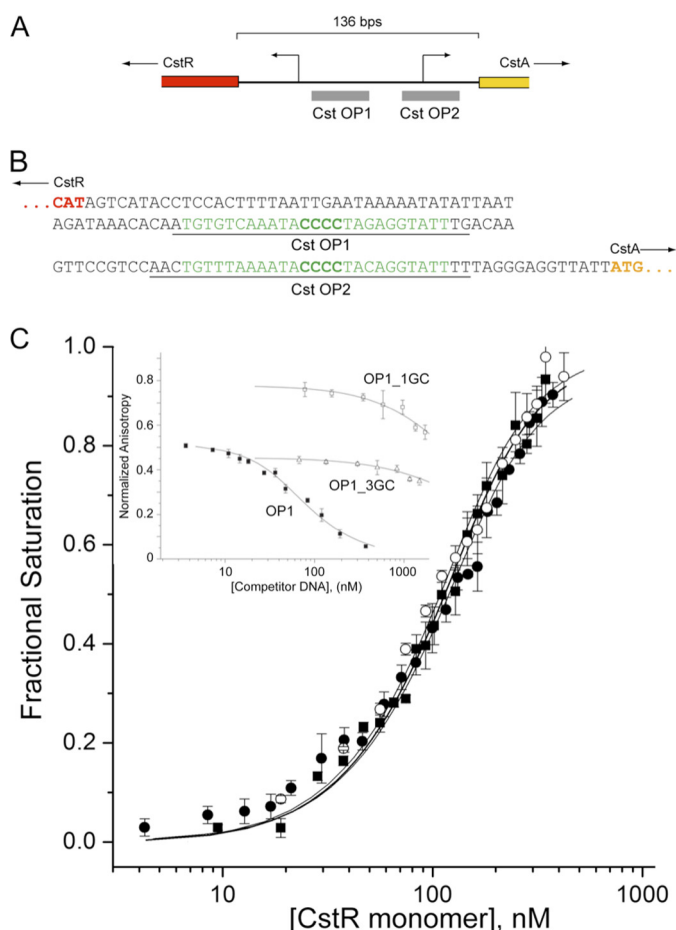


**FIGURE 3. CstR regulates the expression of immediately adjacent genes.** *A*, schematic to scale representation of the genome encompassing locus tags NWMN\_0026 to NWMN\_0029 in *S. aureus* strain Newman in the immediate vicinity of NWMN\_0026.5. Trivial names and the number of amino acid residues of each encoded protein are shown, and the number of nucleotides that separate each ORF, for reference. *TauE*, putative sulfite/sulfonate effluxer; *CstA* and *CstB*, CsoR-like sulfur transferase-regulated genes A and B; *SQR*, putative sulfide:quinone reductase. *SauSQR* exhibits 57% identity to *bona fide* SQR from *Bacillus stercorophilus* (*Bst*) (heavy metal tolerance-2 protein, HMT-2), which has been shown to catalyze sulfide-dependent menaquinone reduction (53). *B*, NWMN\_0027 through NWMN\_0029 form an operon. cDNA from  $\Delta$ *cstR* was subjected to PCR using primers that anneal with the *sqr* and *cstA* genes and amplify a 2.7-kb product. No cDNA (RT) was used as a negative control, whereas chromosomal (*Chrm*) was used as a positive control. *C–F*, analysis of gene expression by quantitative RT-PCR. *C*, NWMN\_0027 through 0029 and *tauE* levels in  $\Delta$ *cstR* relative to wild type. NWMN\_0027 and *tauE* levels in  $\Delta$ *csoR* (*D*) and wild-type (*E*) and in the presence and absence of copper. *F*, expression of *copA* in wild-type and  $\Delta$ *cstR* strains. Results represent the average of three or more independent experiments. \*,  $p < 0.05$  determined by Student's *t* test. N.S., not significant. Error bars = S.D.

four-amino acid fingerprint (11) of X-Cys-X-Cys (where X is any amino acid) that conserves the two Cu(I)-binding cysteines of CsoRs in the X and Z positions, but replaces the key Cu(I) coordinating His ligand (Y) with an Asn (supplemental Fig. S5). This suggests that the protein encoded by NWMN\_0026.5 (*CstR*) cannot function as a Cu(I) sensor. To determine the genes regulated by *CstR*, we created a  $\Delta$ *cstR* derivative and assessed expression of the immediately adjacent genes *tauE*, and NWMN\_0027–0029 by quantitative RT-PCR. Loss of *CstR* gives rise to a massive increase in expression of all four genes

(Fig. 3C). The genetic structure of this loci suggests that NWMN\_0027 through NWMN\_0029 forms an operon, which was confirmed by amplification of a PCR product spanning these genes from cDNA (Fig. 3B).

*CstR* Binds Specifically to Each of Two Operator Sites Situated between the *cstR* and *tauE* Genes—Two strong candidate tandemly repeated *CstR* operator sites, *CstO* OP1 and OP2, were found between the *cstR* and *cstA* genes. These sites were characterized by a run of four consecutive GC-base pairs and flanked by AT-rich regions (Fig. 4, A and B). We first purified



**FIGURE 4. Reduced CstR binds to *cst* operator (*CstO*) sites with high affinity in a manner dependent of the central run of four GC base pairs.** *A*, schematic of the *cstR-cstA* intergenic region highlighting the positions of the two tandem candidate *CstR* operator sites. *B*, nucleotide sequence of the *cstR-cstA* intergenic region, highlighting the OP1 and OP2 operator sequences (green). Underlined bases correspond to the 5'-fluorescein-labeled duplex oligonucleotides used for DNA binding experiments. *C*, *CstR* binding isotherms for OP1 (solid squares, ■), OP2 (open circles, ○), and OP1\_5GC (filled circles, ●) in which an additional GC base pair was inserted into the run of four GC base pairs. Inset, competition dissociation experiments with fluorescein-labeled apo-*CstR*-OP1 complexes with unlabeled wild-type OP1 (solid squares, ■), OP1-GC3 (open triangles, △), and OP1\_1GC (open squares, □) duplexes. Fitted parameters derived from a two-tetramer binding model (15, 16) are compiled in Table 1. Conditions used were pH 7.0, 0.2 M NaCl, at 25.0 °C.

**TABLE 1**

**Equilibrium binding parameters for reduced *S. aureus* CstR or CsoR for fluorescein-labeled Cst OP1 or OP2 DNA duplexes**

Conditions used were 10 mM Hepes, pH 7.0, 0.2 M NaCl, at 25.0 °C.

Protein	DNA	$r_o$	$r_{\text{complex}}$	$K_1^a$	$K_2^a$	$K_{\text{tet}}^a$
					$\times 10^7 \text{ M}^{-1}$	
CstR	OP1	0.129	0.226	0.7 ( $\pm 0.3$ )	55 ( $\pm 5$ )	27 ( $\pm 5$ )
CstR	OP2	0.142	0.221	2.4 ( $\pm 0.1$ )	18 ( $\pm 9$ )	10 ( $\pm 9$ )
CstR	OP1 <sup>b</sup>					6 ( $\pm 5$ )
CstR	OP1_5GC	0.112	0.148	3 ( $\pm 1$ )	13 ( $\pm 4$ )	8 ( $\pm 4$ )
CstR	OP1_3GC <sup>b</sup>					0.5 ( $\pm 0.1$ )
CstR	OP1_1GC <sup>b</sup>					0.09 ( $\pm 0.01$ )
CstR <sup>S-S</sup> , CstR <sub>2</sub> <sup>S-S</sup>	OP1	0.129	0.226 <sup>c</sup>	0.31 ( $\pm 0.03$ )	0.15 ( $\pm 0.02$ )	0.23 ( $\pm 0.03$ )
CstR <sup>S-SMe</sup>	OP1	0.129	0.226 <sup>c</sup>	0.15 ( $\pm 0.04$ )	0.0006 ( $\pm 0.01$ )	0.08 ( $\pm 0.04$ )
Apo-CsoR	OP1	0.130	0.221	6 ( $\pm 2$ )	20 ( $\pm 5$ )	13 ( $\pm 5$ )
Apo-CsoR	<i>Cop</i>	0.092	0.101	0.2 ( $\pm 0.2$ )	5 ( $\pm 6$ )	3 ( $\pm 6$ )
CsoR <sup>S-SMe</sup>	OP1	0.130	0.221 <sup>d</sup>	0.2 ( $\pm 0.1$ )	0.001 ( $\pm 0.02$ )	0.10 ( $\pm 0.02$ )

<sup>a</sup> Determined from a model that assumes two tetramers bind to each operator DNA with step-wise association constants of  $K_1$  and  $K_2$  and  $K_{\text{tet}}$  is the average macroscopic tetramer association constant ( $K_{\text{tet}} = \text{sqrt}(K_1 \cdot K_2)$ ).

<sup>b</sup> Determined by competitive dissociation experiments of fluorescently-labeled protein-OP1 complexes with unlabeled DNA competitors (23).

<sup>c</sup> Fixed to underivatized CstR.

<sup>d</sup> Fixed to underivatized CsoR.

recombinant, fully reduced *Sau* Newman CstR to  $\geq 95\%$  homogeneity from *E. coli*. CstR purified in this way migrates as a mixture of noncovalent dimers, tetramers, and octamers by gel filtration chromatography at 75  $\mu\text{M}$  (monomer), with the primary species apparently octameric (supplemental Fig. S6). The binding of CstR to fluorescein-labeled OP1 and OP2 (see Fig. 4B, underlined sequences) was assessed by anaerobic titration measuring the fractional change in the anisotropy of the fluorescein fluorescence (Fig. 4C). CstR binds with high affinity to each *cst* operator site and the stoichiometry was determined using a model-independent method of analysis of two tetramers per operator, as previously determined for *Mtb* and *Bsu* CsoRs (15, 16) (supplemental Fig. S7). Fitting the binding data to a two-tetramer binding model gives an average tetramer-DNA association equilibrium constant,  $K_{\text{tet}}$  of  $\approx 2.7 \times 10^8 \text{ M}^{-1}$ , pH 7.0, 0.2 M NaCl, at 25 °C, with some evidence for positive cooperativity of stepwise binding of tetramers to the DNA ( $K_2 > K_1$ ) (Table 1).

To assess the DNA binding specificity of Cst OP1, we tested three mutant OP1 duplexes, one of which adds an additional GC base pair, OP1\_5GC, whereas the others replace one (OP1\_3GC) or three (OP1\_1GC) GC base pairs with AT base pairs. The extended OP1\_5GC duplex forms a complex with an affinity within 4-fold of OP1, whereas deletion of one or three GC pairs abolishes specific complex formation (Fig. 4C). These findings reveal that *Sau* CstR possesses sequence- and/or structure-specific DNA binding properties consistent with other CsoR/RcnR regulators (27).

The Cu(I) sensor CsoR binds to the non-cognate Cst OP1 with an affinity similar to that of CstR and  $\approx 4$ -fold more tightly than to the cognate *cop* operator DNA (supplemental Fig. S8). This is not surprising given the similarity of the operator sequences, each of which is characterized by a run of 4–5 GC base pairs flanked by a 2–3 G-C base pairs (supplemental Fig. S9). This binding appears specific and is negatively regulated by Cu(I) (supplemental Fig. S10). However, the CstR regulon in wild-type or  $\Delta\text{csoR}$  strains is unaffected by copper added to the growth medium, indicating that CsoR has no role in CstR-regulated repression (Fig. 3, D–E). Likewise, Cu(I)-mediated depression of CsoR-regulated *copA* expression is unaffected by deletion of the *cstR* gene (Fig. 3F), further evidence that these

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**TABLE 2**

Molecular masses of reaction products obtained upon anaerobic incubation of reduced *S. aureus* CstR or CsoR with various sulfur-containing compounds

The conditions used are: 10 mM Hepes, pH 7.0, 0.2 M NaCl, at 25.0 °C. All 10-fold molar excess of Cys residues unless otherwise indicated, 20 μM protomer.

Protein	Reactant	Molecular mass observed	Assignment	Expected modification	Molecular mass expected <sup>a</sup>
		<i>Da</i>			<i>Da</i>
CstR	No addition	9640.7			9641.2
	Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub>	9640	NR <sup>b</sup>	–SH (×2)	9673.2 (9705.2)
	Na <sub>2</sub> S	9640	NR	–SH (×2)	9673.2 (9705.2)
	Na <sub>2</sub> SO <sub>3</sub>	19280	CstR <sub>2</sub> <sup>S-S</sup>	CstR <sub>2</sub> <sup>S-S</sup>	19280.4
		19312	CstR <sub>2</sub> <sup>S-S-S</sup>	CstR <sub>2</sub> <sup>S-S-S</sup>	19312.4
		19344	CstR <sub>2</sub> <sup>(S-S-S)2c</sup>	CstR <sub>2</sub> <sup>(S-S-S)2c</sup>	19344.4
CsoR	MMTS	9732	–SCH <sub>3</sub> ×2	–SCH <sub>3</sub> (×2)	9733.2
	No addition	11035			11036.6
	Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub>	11035	NR	–SH (×2)	11068.6 (11100.6)
	Na <sub>2</sub> S	11035	NR	–SH (×2)	11068.6 (11100.6)
	Na <sub>2</sub> SO <sub>3</sub> <sup>d</sup>	11035	NR	CsoR <sub>2</sub> <sup>S-S</sup>	22073.2
				CsoR <sub>2</sub> <sup>S-S-S</sup>	22105.2
	MMTS	11128	–SCH <sub>3</sub> ×2	–SCH <sub>3</sub> (×2)	11130.6

<sup>a</sup> Expected masses were calculated as average masses [M] with no modifications at EXPASY.

<sup>b</sup> NR, no reaction.

<sup>c</sup> Both interprotomer cysteine linkages incorporate a sulfur atom in the dimer.

<sup>d</sup> 100-fold excess of reagent.

two regulatory systems function independently of one another in the cell.

*How Is cst Operator DNA Binding by CstR Regulated?*—The *cst* regulon encodes a putative sulfite/sulfonate effluxer TauE (NWMN\_0026) (28) and two of the four candidate rhodanese sulfur transferase domains in *S. aureus* (29) (CstA, NWMN\_0027; CstB, NWMN\_0028), a potential sulfur dioxygenase (CstB, NWMN\_0027) (30), and a predicted sulfide quinone reductase (NWMN\_0029) (see Fig. 3A) (31). These proteins may allow *S. aureus* to utilize thiosulfate (S<sub>2</sub>O<sub>3</sub><sup>2-</sup>) as a sulfur source for cysteine biosynthesis. To assimilate sulfur from thiosulfate, the S-S bond must ultimately be broken, perhaps catalyzed by a rhodanese domain, with the release of sulfite. *S. aureus* cannot metabolize sulfite, a potent oxidant or pro-oxidant that alters thiol-disulfide homeostasis, because it lacks both sulfite reductase and sulfite oxidase. We reasoned that sulfite may be toxic to *S. aureus* and could be effluxed by the CstR-inducible TauE. Growth curves on a chemically defined medium indeed reveal that *S. aureus* is capable of utilizing thiosulfate as a sole sulfur source for cysteine biosynthesis (32), with sulfide far less effective, and sulfite completely inhibitory to growth (supplemental Fig. S11). We therefore hypothesized that derivatization of CstR thiolates by thiosulfate, sulfite, or sulfide, rather than Cu(I) binding, might negatively regulate DNA binding.

To test this, we performed anaerobic incubations of reduced CstR (20 μM protomer) with a 10–100-fold molar excess of sodium thiosulfate or sodium sulfide; these trials failed to yield any products other than the reduced CstR starting material as measured by ESI-MS of full-length proteins (Table 2). In striking contrast, anaerobic incubation with sodium sulfite at a 5–100-fold molar ratio over CstR cysteine resulted in three major oxidized products consistent with the following assignments: disulfide cross-linked dimer, CstR<sub>2</sub><sup>S-S</sup>, and single and double trisulfide CstR<sub>2</sub><sup>S-S-S</sup> cross-linked dimers where an additional sulfur atom bridges one or both cysteine pairs within the dimer (33) (Table 3). An ESI-MS spectrum of the interprotomer disulfide-bridged tryptic peptide obtained from a sulfite-treated CstR sample confirms the CstR<sub>2</sub><sup>S-S</sup> assignment (Fig. 5),

**TABLE 3**

Molecular masses of major species identified by LC-ESI-MS as a result of anaerobic incubation with sodium sulfite of fully reduced CstR

The conditions used were: 10 mM Hepes, pH 7.0, 0.2 M NaCl, at 25.0 °C. The indicated fold-excess is molar excess over Cys residues (20 μM protomer, 40 μM cysteine).

Reaction condition	Molecular mass observed	Assignment	Molecular mass expected
	<i>Da</i>		<i>Da</i>
No reaction	9640.7	CstR <sup>a</sup>	9641.2
	9662.8	CstR + Na <sup>+</sup>	9663.2
+5X SO <sub>3</sub> <sup>2-</sup>	9639.8	CstR	9641.2
	19280.0	CstR <sub>2</sub> <sup>S-S</sup>	19280.4
	19311.8 (+32) <sup>b</sup>	CstR <sub>2</sub> <sup>S-S-S</sup>	19312.4
	19324.7 (+45)	CstR <sub>2</sub> <sup>S-S-S</sup> + 2 Na <sup>+</sup>	19324.4
	19343.7 (+64)	CstR <sub>2</sub> <sup>(S-S-S)×2</sup>	19344.4
+10X SO <sub>3</sub> <sup>2-</sup>	9640.4	CstR	9641.2
	9661.2	CstR + Na <sup>+</sup>	9663.2
	19280.7	CstR <sub>2</sub> <sup>S-S</sup>	19280.4
	19295.1 (+14)	CstR <sub>2</sub> <sup>S-S</sup> + O <sup>c</sup>	19296.4
	19312.4 (+32)	CstR <sub>2</sub> <sup>S-S-S</sup>	19312.4
	19323.4 (+44)	CstR <sub>2</sub> <sup>S-S</sup> + 2 Na <sup>+</sup>	19324.4
	19334.0 (+53)	CstR <sub>2</sub> <sup>S-S-S</sup> + Na <sup>+</sup>	19334.4
	19357.6 (+77)	CstR <sub>2</sub> <sup>S-S-S</sup> + 2 Na <sup>+</sup>	19356.4
	19366.5 (+86)	CstR <sub>2</sub> <sup>(S-S-S)×2</sup> + Na <sup>+</sup>	19366.4
+100X SO <sub>3</sub> <sup>2-</sup>	9640.1	CstR	9641.2
	19280.0	CstR <sub>2</sub> <sup>S-S</sup>	19280.4
	19311.3 (+31)	CstR <sub>2</sub> <sup>S-S-S</sup>	19312.4
	19332.7 (+43)	CstR <sub>2</sub> <sup>S-S-S</sup> + Na <sup>+</sup>	19334.4
	19343.0 (+63)	CstR <sub>2</sub> <sup>(S-S-S)×2</sup>	19344.4

<sup>a</sup> CstR, reduced CstR monomer (starting material).

<sup>b</sup> Mass increase relative to oxidized dimer (CstR<sub>2</sub><sup>S-S</sup>).

<sup>c</sup> One disulfide linkage, with one sulfenic acid (–SOH) on the opposite side of the dimer; tentative assignment.

which is absent in untreated, reduced CstR starting material. This mixture of CstR<sub>2</sub><sup>S-S</sup> and CstR<sub>2</sub><sup>S-S-S</sup> binds weakly to the *cst* operator OPI, thus revealing that oxidation is strongly negatively regulatory (Fig. 6). In contrast, the Cu(I) sensor CsoR is unreactive toward sodium sulfite, even at a 100-fold molar excess of reagent (Table 2).

Anaerobic incubation of reduced CstR with a 10-fold molar excess of the thiol modifying reagent MMTS quantitatively converts CstR to the doubly S-methylated derivative, denoted CstR<sup>SSMe</sup> (Table 2). The mixed disulfide RS-SCH<sub>3</sub> is the methyl analog of a cysteine persulfide (RS-SH) as well as other mixed disulfides (RS-SR') that could form with low molecular weight cellular thiols, and is thus of interest. CstR<sup>SSMe</sup> also binds

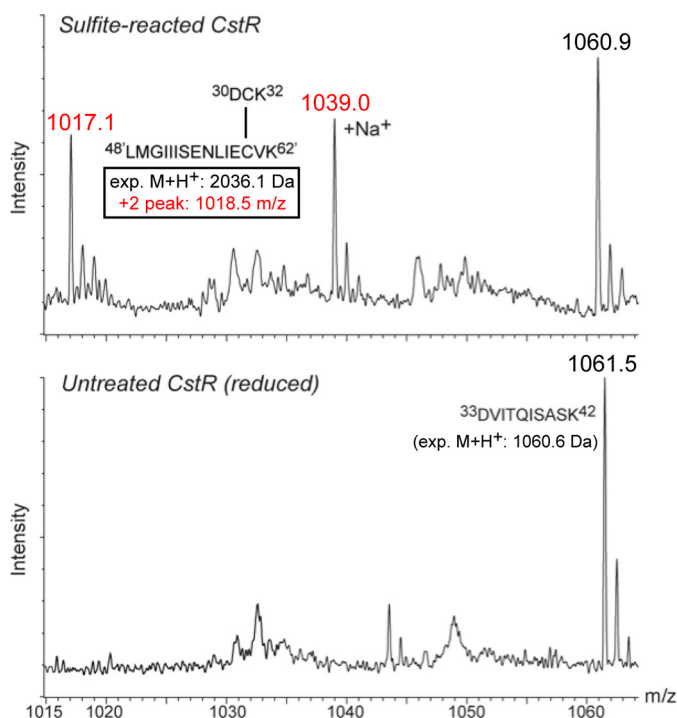


FIGURE 5. A portion of an ESI-MS of a tryptic digest of a sample of CstR reacted anaerobically with  $\times 10$  sulfite for 48 h versus one left untreated for 48 h. The peptide indicated is only found in the sulfite-reacted sample and has a molecular weight consistent with a disulfide cross-link between tryptic peptides corresponding to those containing the N-terminal Cys<sup>31</sup> (<sup>30</sup>DCK<sup>32</sup>) and C-terminal Cys<sup>60</sup>, from opposite subunits within the dimer (expected monoisotopic  $M+H^+$  masses indicated).

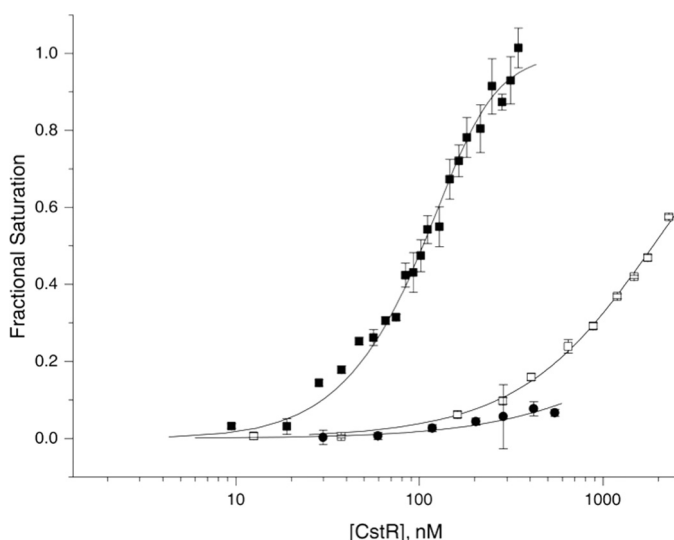


FIGURE 6. The DNA binding activity of CstR is negatively regulated by covalent cysteine disulfide and trisulfide derivatization by sodium sulfite. Unmodified fully reduced (solid squares, ■), sulfite-treated (open squares, □), and MMTS-reacted (solid circles, ●) CstRs were titrated into a solution of 10 nM Cst OP1 DNA and anisotropy ( $r_i$ ) was recorded in triplicate. Each data point represents the mean  $\pm$  S.D. following each  $i$ th addition of protein, following normalization to the starting ( $r_o$ ) and ending ( $r_{\text{complex}}$ ) values of the anisotropy of the underivatized CstR complex. The solid line is a fit to a two-tetramer binding model, with the anisotropy of the 1:1 complex fixed to  $0.5 \times (r_{\text{complex}} - r_o)$ . Fitted parameters are compiled in Table 1. Conditions used were 10 mM HEPES, pH 7.0, 0.2 M NaCl, at 25.0 °C.

weakly to the OP1 DNA, revealing strong inhibition of CstR binding upon *S*-methylation, like that observed for oxidation (Fig. 6). Treatment of the Cu(I) sensor CsoR under the same

conditions yielded no reaction, although quantitative derivatization was possible at a 100-fold molar excess of MMTS (Table 2). CsoR derivatized in this way also results in weak non-cognate *cst* operator binding (supplemental Fig. S10).

## DISCUSSION

In this work, we characterize two transcriptional regulatory responses controlled by paralogous regulators from the CsoR/RcnR family of proteins. One is a Cu(I)-stress response under control of a CsoR (13). The other encodes proteins that we predict will be required for sulfur assimilation from an inorganic sulfur source, thiosulfate, and is under control of a new regulator, termed CstR. CstR is the first characterized member of a subfamily of CsoR/RcnR regulators that do not appear to respond to transition metal stress, but are characterized by a high reactivity toward sulfite and the sulfur-modifying agent MMTS. Our data support the hypothesis that the interprotomer cysteine pair in CstR relative to CsoR readily forms a disulfide or trisulfide bond across this interface. This in turn drives a conformational change in the oligomer that reduces the affinity of CstR for the operator DNA, thus leading to transcriptional depression of the operon *in vivo*. CsoR, on the other hand, is not effectively derivatized by sulfite (Table 2).

**Sulfur Assimilation in *S. aureus***—*S. aureus* is characterized by a unique thiol metabolism and is strongly restricted in its ability to obtain inorganic sulfur to make cysteine (32, 34). Sulfate is the preferred bacterial sulfur source but all sequenced *S. aureus* strains lack adenosine 5'-phosphosulfate reductase and thus cannot utilize sulfate as a sulfur source. Sulfite and alkyl-sulfonates, e.g. taurine, are likewise not assimilated and sulfite cannot be metabolized (35, 36). Organic sulfur sources, including glutathione and homocysteine, are preferred sources of cysteine, but both must be acquired by *S. aureus* from the host.

We establish here that *S. aureus* strain Newman is able to grow on thiosulfate as the sole sulfur source (36). Thiosulfate is a major excreted sulfur-containing compound in humans, and is thought to be generated by the oxidation of bacterially derived H<sub>2</sub>S by an essential mitochondrial sulfur dioxygenase in mucosal cells to protect against sulfide toxicity (30). Although the bioavailability of thiosulfate in *S. aureus* abscesses is not known, recent findings reveal that colonization of the gastrointestinal epithelium by *Salmonella* gives rise to an inflammatory burst that oxidizes thiosulfate to tetrathionate (S<sub>4</sub>O<sub>6</sub><sup>2-</sup>), which is used as an alternative terminal electron acceptor for respiration under anaerobic conditions (37). Although *S. aureus* lacks the tetrathionate respiratory complex, a major form of oxidized sulfur in inflamed tissues may well be tetrathionate. The physiological inducer of the *cst* regulon is as yet unknown.

One hypothesis is that the *cst* genes are induced by any condition that specifically alters thiol-disulfide homeostasis, including, but not limited to, sulfite stress. This would result in facile oxidation of the regulatory cysteines in CstR or formation of mixed disulfides with major low molecular weight thiols in *S. aureus*, e.g. bacillithiol (34, 38), coenzyme A, or cysteine, each of which is expected to drive transcriptional derepression on the basis of the observed weak DNA binding activity of *S*-methylated CstR (Fig. 6). General perturbation of thiol-disulfide homeostasis would signal to the organism to obtain sulfur for



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cysteine and/or bacillithiol biosynthesis from all available sources, including inorganic thiosulfate, particularly under conditions of low host-derived organic sulfur.

Another possible inducer of *cst* expression is the sulfonated  $\beta$ -amino acid taurine or an as yet unidentified taurine metabolite. *S. aureus* does not encode a taurine repressor TauR; thus, taurine might also be indirectly sensed by CstR as sulfite or another metabolite (39). Taurine is a highly abundant amino acid in mammals and a dedicated  $\text{Na}^+$ -dependent uptake system for taurine is encoded by *ssuBAC* in *S. aureus* (35). However, *S. aureus* does not encode TauD or SsuDE and it is therefore unlikely that taurine is oxidized to aminoacetaldehyde with the release of sulfite. The other known alternative route of degradation of taurine is release of the  $\text{NH}_4^+$  with the formation of negatively charged sulfoacetaldehyde (40). It is interesting to note that both routes of taurine degradation give rise to aldehydes, highly electrophilic carbon atoms that may well be quite reactive with CstR cysteines to form thioesters. Indeed, formaldehyde stress in *E. coli* has been proposed to be sensed by a CsoR family member FrmR, the mechanism of which is unknown (11, 41), and in *B. subtilis*, bacillithiol protects cells against electrophile stress (42).

**DNA Binding and Cross-transcriptional Regulation of CsoR Versus CstR**—The mechanism of operator recognition by these novel disc-shaped CsoR/RcnR family transcriptional repressors is not fully understood. Previous work proposed two general types of DNA operators, termed type 1 and type 2, which differ in the organization and number of GC base pair tracts (27) but seem to incorporate features of “shape selective” or “indirect readout”-based recognition of a pair of A-B helical junctions, and specific features of the flanking base pairs.

The type 2 operators possess two short stretches of GC base pairs (one block of 2–3 base pairs, another of 4–5 base pairs, often of opposite strand polarity) separated by 3–4 base pairs, and flanked by a nearly conserved palindromic ATA sequence sometimes in the context of a larger inverted repeat (supplemental Fig. S9). Both *cop* and *cst* operators in *S. aureus* as well as that of *B. subtilis* CsoR (15, 20) and all target sequences for *M. tuberculosis* RicR (6) conform to this consensus sequence. This places two predicted A-B helical junctions one helical turn (10–11 base pairs) apart (27), and this may facilitate the binding of two tetramers at these junctions, as validated for *Bsu* CsoR (15) and CstR here (supplemental Fig. S7). Such a model also explains why addition of a fifth consecutive GC base pair to Cst\_OP1 is energetically nearly silent (Table 1); this simply increases the spacing between flanking palindromes from 10 to 11 base pairs, exactly as found in the *Sau* *cop* operator (supplemental Fig. S9). Reducing the number of consecutive GC base pairs to three (in CstOP1\_3GC) may well change the structure of the operator, abolishing shape selective recognition by CstR.

The *Sau* *cop* operator sequence contains one consensus operator (Fig. 1), and the divergently transcribed *cstR-cstA* intergenic region contains two consensus operators, each of which binds two CstR tetramers (Fig. 4). As a result, tandem operators in the *cstR-cstA* intergenic region can therefore accommodate four tetramers (two octamers) in total, and thus can potentially adopt a higher order, strongly repressing a nucleoprotein complex that is unlikely to occur in the single

operator *cop* sequence. Perhaps CsoR is not able to assemble such a higher order structure and thus does not strongly repress the *cst* operon in  $\Delta$ *cstR* cells (Fig. 3) despite binding to a single consensus operator site with an affinity equal to or better than that of cognate CstR (Table 1).

***S. aureus* CsoR and Copper Toxicity**—Control of copper homeostasis in bacteria is particularly important because nearly all prokaryotes, outside of photosynthetic bacteria, appear to lack an obligatory intracellular cytoplasmic copper requirement (43–45). Uncomplexed copper ions are extremely toxic, attributed to an autocatalytic Haber-Weiss reaction (46) or displacement of iron from solvent-exposed iron-sulfur clusters in proteins (47). As a result, cytoplasmic bioavailable copper levels must be strictly controlled and are proposed to be buffered at concentrations in the  $10^{-18}$ – $10^{-21}$  M range despite a total concentration in the low micromolar range (48, 49).

Here, we establish that *S. aureus* CsoR is a Cu(I) sensing CsoR, binds Cu(I) with an affinity ( $K_{\text{Cu}}$ ) of  $10^{18} \text{ M}^{-1}$ , and possesses structural, allosteric and Cu(I) coordination features characteristic of *Mtb* and *Bsu* CsoRs (15, 16). A major point of departure from other bacterial systems is that the sensor is genetically unlinked from the expression of the *copA* and *copZ* resistance genes, thus making its identification and functional role more difficult to establish; furthermore, *S. aureus* CsoR is unique in that it oligomerizes beyond the stable tetramer assembly state under the conditions used here (supplemental Figs. S12 and S13). The significance of this finding is unknown.

Microarray experiments in *S. aureus* SH1000 reveal that the expression of CsoR is not strongly induced by copper stress, unlike the situation in *M. tuberculosis* (13, 50); as a result, constitutively expressed CsoR might play a role in buffering “free” Cu(I) in *S. aureus* in the  $\approx 10^{-18}$  M range. On the other hand, the gene encoding the likely copper chaperone CopZ just downstream from *copA* (Fig. 1A) is induced by copper (24, 25), but may not be co-transcribed with *copA* (24). Although it is not yet known whether the transcription of *copZ* is also regulated by CsoR, inspection of the intergenic region between *copA* and *copZ* does not reveal an obvious candidate CsoR binding site like that found in the *copA* operator-promoter region, nor have we determined if *copZ* transcription is altered in the  $\Delta$ *csoR* strain.

**Implications for Bacterial Virulence**—It is interesting to note that the *cst* operon core (*tauE-cstR-cstAB*) and both CstR operator binding sites (Fig. 3) are duplicated in the genomes of major antibiotic-resistant *S. aureus* strains (N315, COL, and Mu50) (51). In contrast, the *tauE* gene is lost in at least one methicillin-sensitive *Sau* strain (MSSA) (51). These genomics data are consistent with the idea that this putative sulfur assimilation regulon provides a growth advantage to *Sau* during infection of the host. Recent findings suggest that alterations in sulfur metabolism influence the ability of *Sau* to form biofilms, an essential feature of its ability to thrive both inside and outside of the host (32). Microarray experiments carried out on mid-log liquid cultures of *S. aureus* SH1000 reveal that copper stress, sufficient to induce the CsoR-dependent expression of *copA* and perhaps other genes that function in oxidative stress resistance (25), negatively regulates biofilm formation via repression of the *sae* and *agr* regulons (52). Thus, CsoR and

CstR-related stress response pathways may be physiologically linked by a connection to alteration in biofilm formation and oxidative stress and ultimately viability in the vertebrate host.

*Acknowledgments*—Portions of this research were carried out at the Stanford Synchrotron Radiation Lightsource operated by Stanford University on behalf of the United States Department of Energy, Office of Basic Energy Sciences. The SSRL Structural Molecular Biology Program is supported by the Department of Energy, Office of Biological and Environmental Research, and the National Institutes of Health, National Center for Research Resources Biomedical Technology Program.

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