Supporting Information

The CsoR-like sulfurtransferase repressor (CstR) is a persulfide sensor in *Staphylococcus aureus*

Luebke *et al.*

This file contains **Supporting Tables S1-S5** and **Supporting Figures S1-S8.**

Table S1. Summary of tetrameric CstR association equilibrium constants (K_{tet}) for *cst* OP1 obtained from fluorescence anisotropy titrations obtained in this study and others. All experiments performed with fluorescently labeled *cst* OP1 unless otherwise noted. Conditions: 10 mM HEPES, pH 7.0, 200 mM NaCl, 25.0 ˚C.

Protein	K_{tet} (x10^7) M^{-1}
$CstR^{RSH}$	6.3 (±0.5)[a]
	25 (±5)[b]
	6 (±5)[b*]
	10 (±9)[b†]
	7.4 (±1.8)[c]
CstR + NaHS	0.06 (±0.05)[c]
CstR + Na_2S_4	0.03 (±0.03)[c]
CstR:Cu(I)	4.3 (±1.7)[c]
C31A CstR	3.3 (±0.7)[a]
S-Me C31A CstR[§]	2.0 (±0.5)[a]
C60A CstR	4.2 (±1.0)[a]
S-Me C60A CstR[§]	0.31 (±0.03)[a]

[a]From reference (Luebke *et al.*, 2013)
[b]From reference (Grossoehme *et al.*, 2011)
[c]This study
[*]Determined from competitive dissociation with unlabeled competitors
[†]CstR-*cst* OP2 binding affinity
[§]Preprared by reaction with methylmethanethiolsulfonate (MMTS) (Luebke *et al.*, 2013)

Table S2. Summary of LC-ESI-MS deconvoluted masses following reaction with the indicated compound. All mass shifts are relative to either the reduced ($\mathrm{CstR^{RS\text{-}H}}$) or disulfide bonded form of CstR ($\mathrm{CstR_2^{RS\text{-}SR'}}$) as indicated by (-). Observed masses in *italics* represent masses observed in controls and in the starting masses.

Protein	M_r Observed (Da)	Mass Shift	Assignment	M_r Expected (Da)
CstR Control	*9640.2*	-	$CstR^{RS\text{-}H}$	*9641.2*
	9661.3	*21.1*	$CstR^{RS\text{-}H} + Na$	*9663.2*
	19279.3	-	$CstR_2^{RS\text{-}SR'}$	*19280.4*
CstR + 5x NaHS	*9640.0*	-	$CstR^{RS\text{-}H}$	*9641.2*
	9662.5	*22.5*	$CstR^{RS\text{-}H} + Na$	*9663.2*
	19279.7	-	$CstR_2^{RS\text{-}SR'}$	*19280.4*
	19311.7	32.0	$\mathrm{CstR_2^{RS\text{-}S\text{-}SR',\ R''S\text{-}SR'''}}$	19312.5
	19342.8	63.1	$\mathrm{CstR_2^{RS\text{-}S\text{-}SR',\ R''S\text{-}S\text{-}SR'''}}$	19344.5
	19372.5	92.8	$\mathrm{CstR_2^{RS\text{-}S\text{-}S\text{-}SR',\ R'S\text{-}S\text{-}SR'''}}$	19376.6
	19403.7	124.0	$\mathrm{CstR_2^{RS\text{-}S\text{-}S\text{-}SR',\ R''S\text{-}S\text{-}S\text{-}SR'''}}$	19408.7
CstR + 5x Na_2S_4	*9642.5*	-	$CstR^{RS\text{-}H}$	*9641.2*
	19282.0	-	$CstR_2^{RS\text{-}SR'}$	*19280.4*
	19313.9	31.9	$\mathrm{CstR_2^{RS\text{-}S\text{-}SR',\ R''S\text{-}SR'''}}$	19312.5
	19346.5	64.5	$\mathrm{CstR_2^{RS\text{-}S\text{-}SR',\ R''S\text{-}S\text{-}SR'''}}$	19344.5
	19378.9	96.9	$\mathrm{CstR_2^{RS\text{-}S\text{-}S\text{-}SR',\ R'S\text{-}S\text{-}SR'''}}$	19376.6
	19410.0	128.0	$\mathrm{CstR_2^{RS\text{-}S\text{-}S\text{-}SR',\ R''S\text{-}S\text{-}S\text{-}SR'''}}$	19408.7
CstR + 5x NaHS (PO_4^{3-} + EDTA)	*9640.1*	-	$CstR^{RS\text{-}H}$	*9641.2*
	9662.4	*22.3*	$CstR^{RS\text{-}H} + Na$	*9663.2*
	19279.0	-	$CstR_2^{RS\text{-}SR'}$	*19280.4*
CstR + 5x Na_2S	*9640.0*	-	$CstR^{RS\text{-}H}$	*9641.2*
	9662.4	*22.4*	$CstR^{RS\text{-}H} + Na$	*9663.2*
	9684.5	*44.5*	$CstR^{RS\text{-}H} + 2Na$	*9665.2*
	19279.8	-	$CstR_2^{RS\text{-}SR'}$	*19280.4*
CstR + 5x GSSG	*9640.0*	-	$CstR^{RS\text{-}H}$	*9641.2*
	9662.2	*22.2*	$CstR^{RS\text{-}H} + Na$	*9663.2*
	9683.5	*43.5*	$CstR^{RS\text{-}H} + 2Na$	*9665.2*
	19280.0	-	$\mathrm{CstR_2^{RS\text{-}SR'}}$	19280.4
	19302.0	22	$\mathrm{CstR_2^{RS\text{-}SR'} + Na}$	19302.4
CstR + 5x GSH	*9640*	-	$CstR^{RS\text{-}H}$	*9641.2*
	9662.3	*22.3*	$CstR^{RS\text{-}H} + Na$	*9663.2*
	19280.1	-	$CstR_2^{RS\text{-}SR'}$	*19280.4*
CstR + 5x GSSH	*9639.0*	-	$CstR^{RS\text{-}H}$	*9641.2*
	9671.1	32.1	$\mathrm{CstR^{RS\text{-}SH}}$	9673.3
	9702.2	63.2	$\mathrm{CstR^{RS\text{-}SSH}}$	9705.3

19277.1	-	$CstR_2^{RS\text{-}SR'}$	19280.4
19308.4	31.3	$CstR_2^{RS\text{-}S\text{-}SR', R''S\text{-}SR'''}$	19312.5
19339.9	62.8	$CstR_2^{RS\text{-}S\text{-}SR', R''S\text{-}S\text{-}SR'''}$	19344.5
19373.6	96.5	$CstR_2^{RS\text{-}S\text{-}S\text{-}SR', R'S\text{-}S\text{-}SR'''}$	19376.6

Table S3. Summary of LC-ESI-MS deconvoluted masses following reaction with either NaHS or Na_2S_4. All mass shifts are relative to either the reduced ($CsoR^{RS\text{-}H}$) or disulfide bonded form of CsoR ($CstR_2^{RS\text{-}SR'}$) as indicated by (-). *Italics* represent species present control samples.

Protein	M_r Observed (Da)	Mass Shift	Assignment	M_r Expected (Da)
CsoR + 25x NaSH	*11035.5*	-	$\mathit{CsoR}^{RS\text{-}H}$	*11036.6*
	11057.6	*22.1*	$\mathit{CsoR}^{RS\text{-}H} + \mathit{Na}$	*11056.6*
CsoR + 5x Na_2S_4	*11037.7*	-	$\mathit{CsoR}^{RS\text{-}H}$	*11036.6*
	11069.2	31.5	$CsoR^{RS\text{-}SH}$	11068.7
	11101.0	63.3	$CsoR^{RS\text{-}SH}$, $CsoR^{R'S\text{-}SH}$	11100.7
	11149.6	111.9	$CsoR^{RS\text{-}SSH}$, $CsoR^{R'S\text{-}SH}$	11132.8
	22075.5	-	$CsoR_2^{RS\text{-}SR'}$	22071.2
	22106.0	30.5	$CsoR_2^{RS\text{-}S\text{-}SR'}$	22103.2
	22138.4	62.9	$CsoR_2^{RS\text{-}S\text{-}SR', R''S\text{-}S\text{-}SR'''*}$	22135.2
	22170.0	94.5	$CsoR_2^{RS\text{-}S\text{-}S\text{-}SR', R'S\text{-}S\text{-}SR'''}$	22167.3
	22202.9	127.4	$CsoR_2^{RS\text{-}S\text{-}S\text{-}SR', R''S\text{-}S\text{-}S\text{-}SR'''}$	22199.3
	22234.3	158.8	$CsoR_2^{RS\text{-}S\text{-}S\text{-}S\text{-}SR', R''S\text{-}S\text{-}S\text{-}SR'''}$	22231.3
	22267.1	191.6	$CsoR_2^{RS\text{-}S\text{-}S\text{-}S\text{-}SR', R''S\text{-}S\text{-}S\text{-}S\text{-}SR'''}$	22263.3
	22299.2	223.7	$CsoR_2^{RS\text{-}S\text{-}S\text{-}S\text{-}S\text{-}SR', R''S\text{-}S\text{-}S\text{-}S\text{-}SR'''}$	22295.4
	22331.4	255.9	$CsoR_2^{RS\text{-}S\text{-}S\text{-}S\text{-}S\text{-}SR', R''S\text{-}S\text{-}S\text{-}S\text{-}S\text{-}SR'''}$	22327.4
	22363.2	287.7	$CsoR_2^{RS\text{-}S\text{-}S\text{-}S\text{-}S\text{-}SR', R''S\text{-}S\text{-}S\text{-}S\text{-}S\text{-}SR'''} + S$	22359.4

[a]No observed reaction of CsoR with 5x NaHS.

Table S4. Monoisotopic masses of di-, tri-, and tetrasulfide cross-linked parent ions in the +4 charge state observed by high-resolution tandem mass spectrometry. Asterisks (*) indicate masses that were assigned as sulfur as opposed to two oxygen atoms based on the fragmentation pattern of the corresponding peptide (see **Fig. 4**, main text, and **Fig. S4**).

Modification	Monoisotopic Mass (Da)	
	Calculated	Observed
RS-SR'	946.978	946.977
+ O	950.977	950.976
+ 2 O	954.976	954.969*
RS-S-SR'	954.972	954.969
+ 2 O	962.969	962.963*
RS-S-S-SR'	962.965	962.963

Table S5. Primer sequences used in this study.

Primer	**Direction**	**Sequence**
For construction of S. aureus Newman cst deletion strains		
Δ*tauE* 5'	F	GGGGACCACTTTGTACAAGAAAGCTGGGTGTTACATCTTTTCCAGCAGCTCTAGCACC
	R	ACTGCCCGGGAATATCCATTACTTACTTTTCACCAATAAGTTTACAGC
Δ*tauE* 3'	F	ACTGCCCGGGCTCATTTTATAAAACTAATCCTTTTAACTCAGACAGC
	R	GGGGACAAGTTTGTACAAAAAAGCAGGCTGAAGAAGGATATATAAAAGAAATAGTAGGAAGATTGCC
Δ*cstA* 5'	F	ACTGCCCGGGGTATTGTTTCATAATAACCTCCCTAAAAATACCTG
	R	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCCTATTGGAGCAAATATAATACTCCCTATTATAAGG
Δ*cstA* 3'	F	GGGGACCACTTTGTACAAGAAAGCTGGGTAATGATTCTGGAAAATCTTGATAGCCTTC
	R	ACTGCCCGGGTTTATCTAATTAAATCTATTAATAAAAGGAGTTGTTATCATG
Δ*cstB* 5'	F	TTATTTTAATGATTCTGGGTAAAACTGTTTAAAAAACATGATAACAACTCC
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTGACGTTAGAACAGAAGAGGAGTATGCATTAGG
Δ*cstB* 3'	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCAGTAACAATAGGGCATGAAGTATAACCATC
	R	TTTTTTAAACAGTTTTACCCAGAATCATTAAAATAATTTAAGGATGTGG
Δ*SQR* 5'	F	TAATCCTTTTAGCATGCCAATTTGATAATGCTTATTCATTTTTTCCAC
	R	GGGGACCACTTTGTACAAGAAAGCTGGGTGCATACTCCAGGACACACGCCTG
Δ*SQR 3'*	F	GGGGACAAGTTTGTACAAAAAAGCAGGCTAACTATTAGATCTATTGAGATTGCATTTATCATTG
	R	AATAAGCATTATCAAATTGGCATGCTAAAAGGATTAATATAATAAAGTAC
For construction of pOS1 complementation plasmids		

CstR	F	GCGCGCATATGAATTATGATAAAAAAATGATTAATC
	R	GCGCGGGATCCTTACTTACTTTTCACCAATAAGTTTAC
CstA	F	ACGTTACATATGAAACAATACGGAGAAAAGTTTATC
	R	GGGATGGATCCTTAGATAAATAAATTATGATTCGC
CstB	F	CCGCTCGAGTTTTTTAAACAGTTTTACGATAATCATTTATCTCAAGC
	R	CGCGGATCCTTATTTTAATGATTCTGGAAAATCTTGATAGCC
SQR	F	CGCATGCTCGAGGAAAAAATGAATAAGC
	R	CGCGGATCCTTATATTAATCCTTTTAGCATG
CsoR	F	GATAAATACAATTGAGGTGAACATATGGGATATAGTATAGTAAGGAATG TAAATG
	R	CCCCTTGTTTGGATCCTCGAGCATATTAGTCTTTAATCAATTTTTGAAAAG TCAC
For construction of CstR cysteine mutants		
C31A	F	GGAGGAAGGAAAAGAC*TGT*AAAGATGTCATTAC
	R	GTAATGACATCTTT*ACA*GTCTTTTCCTTCCTCC
C60A	F	GGAGGAAGGAAAAGAC*GC*TAAAGATGTCATTAC
	R	GTAATGACATCTTTA*GC*GTCTTTTCCTTCCTCC
For insertion of Xho1 site in pOS1 plasmid		
pOS1 XhoI Quikchange	F	GGTGAACATATGCTCGAGTCTAGAGGATCCAAACAAGGGGG
	R	CCCCCTTGTTTGGATCCTCTAGACTCGAGCATATGTTCACC
For Sequencing pOS1-pLgt Plasmids		
Seq.	F	TAAGAAGAGATGTAAGAGTAG
	R	GATAGAAAAAGGGGG
For qRT-PCR experiments		
16S rRNA	F	GCTGCAGCTAACGCATTAAGCACT
	R	TTAAACCACATGCTCCACCGCTTG
TauE	F	GCACCACCTGCACCTACTATAC
	R	TGAAACCTAGTTCCGAAAAGTC
CstR	F	CGTATTAATAGAATACAAGGGCAACTAAATG
	R	CTCTTTGATGCACTTATTTGTGTAATGACATC
CstA	F	GAAGAAGAAAACGATAGTTATG
	R	GGCCCTGGACATTGAAGACC
CstB	F	AGCGCGAAACAAATGTATGA
	R	CTTTACCACAAGGGCTTCCA
SQR	F	TGTGATATATGCAACGCCAAA
	R	CCGTCGATTTCAACAAGGTT
CopA	F	GCTGCAGCTAACGCATTAAGCACT
	R	TTAAACCACATGCTCCACCGCTTG
For fluorescence anisotropy titrations		
cst OP1	F	ATGTGTCAAATACCCCTAGAGGTATTTG
	R	F-CAAATACCTCTAGGGGTATTTGACACAT

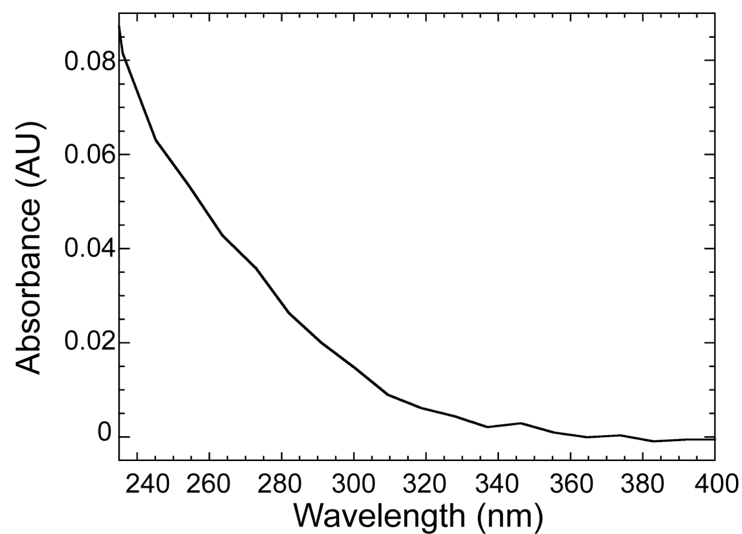

Figure S1. CstR forms a complex with Cu(I). Apoprotein (20 μM protomer)-subtracted UV-Vis spectrum of a 1:1 Cu(I):CstR mixture. This spectrum is characterized by intense absorption from S^{-} to Cu(I) ligand-to-metal or metal-to-ligand charge transfer transitions in the UV region (Liu *et al.*, 2007, Ma *et al.*, 2009) consistent with Cu(I)-thiolate coordination bonds.

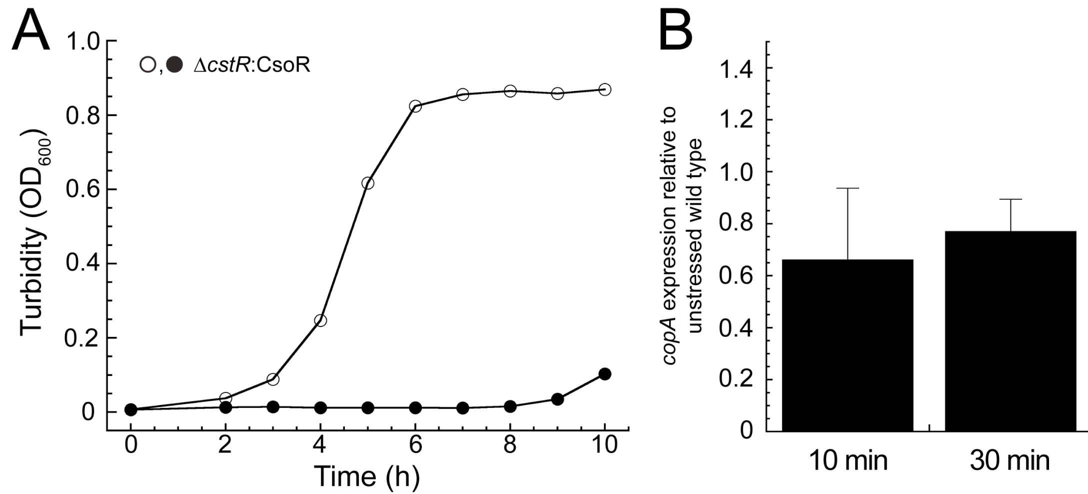

Figure S2. Δ*cstR S. aureus* complemented with *csoR* (Δ*cstR*:CsoR) does not recover normal growth and *copA* is not induced under NaHS stress in WT cells. (A) Δ*cstR* strain transformed with the pOS1 plasmid expressing the copper sensor CsoR from *S. aureus* strain Newman (Grossoehme *et al.*, 2011) does not complement the severe growth phenotype observed for the Δ*cstR* strain (see **Fig. 1B**, main text) in the presence of 0.2 mM NaHS (*filled* circles) vs. absence of added NaHS (*open* circles). Cultures were grown in HHMw+TS growth medium as described in the main text. **(B)** qRT-PCR of *copA* mRNA in WT *S. aureus* following addition of 0.2 mM NaHS stress is not induced, further establishing no *in vivo* crosstalk between CsoR and CstR regulatory pathways. *copA* is under the control of CsoR (Grossoehme *et al.*, 2011). Aliquots were removed before and 10 and 30 min post addition of 0.2 mM NaHS at OD_{600}≈0.2 and qRT-PCR performed as described (see Experimental Procedures)

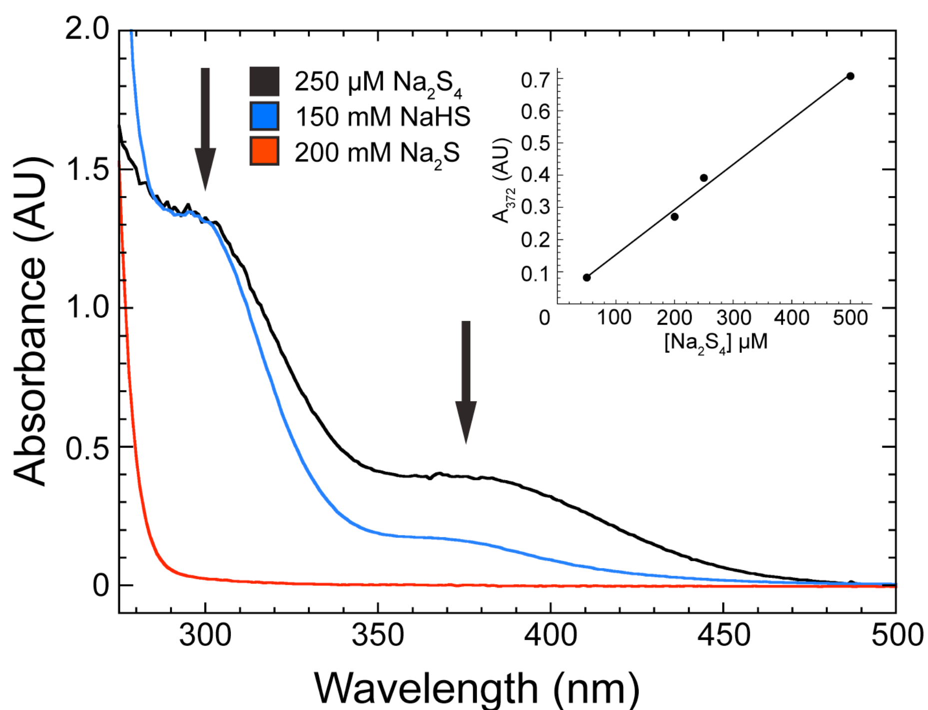

Figure S3. UV Vis spectra of sulfur sources used in this study. Na_2S_4 (black), NaHS (blue), and Na_2S (red) were prepared anaerobically in fully degassed 10 mM Tris-HCl at pH 8.0 containing 1 mM EDTA. The commercial preparation of commercial NaHS (see Experimental Procedures, main text) used here is contaminated with detectable (0.3%) polysulfide as determined by a Na_2S_4 standard curve (*inset*) and gives off a light yellow hue. The yellow coloring is not present in crystalline Na_2S (*red* curve). Arrows indicate locations of characteristic disulfide peaks at 300 and 372 nM (Debiemme-Chouvy *et al.*, 2004, Greiner *et al.*, 2013).

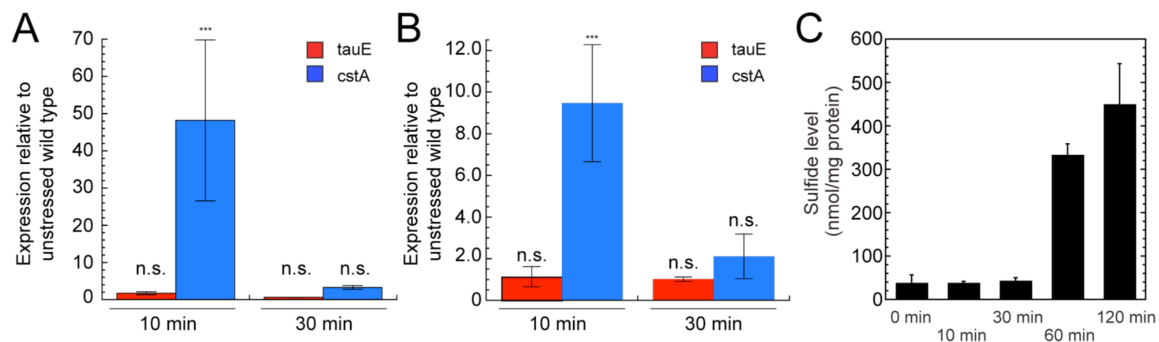

Figure S4. Polysulfide (Na_2S_4) and disodium sulfide (Na_2S) induce the *cst* operon. Wild-type (WT) *S. aureus* cultures grown to an OD_{600} of ≈0.2 and challenged with 25 μM Na_2S_4 **(A)** or 0.15 mM Na_2S **(B)**. Aliquots were removed before and 10 and 30 min post addition of Na_2S_4 or Na_2S. RT-PCR was performed as described (see Experimental Procedures) (*** indicates $p < 0.001$). (**C**) Sulfide concentrations from t=0 to t=120 min induction of the *cst* operon with 25 μM Na_2S_4. Although the kinetics of intracellular HS^- accumulation are slower than observed upon addition of NaHS to cells (see **Fig. 4A**) sulfide ultimately accumulates to the same level of ≈400 nmol mg^{-1} protein.

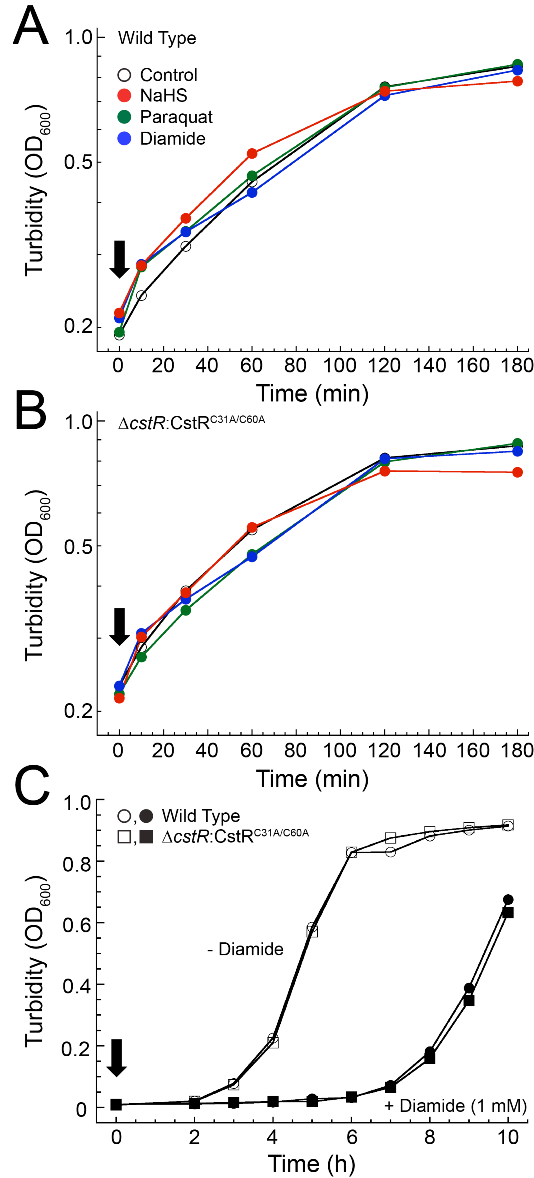

Figure S5. ***Staphylococcus aureus*** **growth rates are not impacted by addition of oxidative stressors and growth under diamide stress is not** ***cst*****-operon dependent.** Addition of *cst* operon inducers NaHS or diamide and non-inducers have no growth phenotype when added to mid-log cells (marked by the arrow, OD_{600}≈0.2). Both WT (**A**) and Δ*cstR*:CstR$^{C31A/C60A}$ (**B**) strains were tested with the addition of 0.2 mM NaSH (*red*), 1.0 mM diamide (*blue*), or 25 nM paraquat (*green*) vs. untreated cells (*black*). (**C**) Diamide was added to a final concentration of 1 mM at the beginning of the growth (marked by the arrow, *filled* symbols) induces a growth phenotype that is equivalent in WT (circles) and Δ*cstR*:CstR$^{C31A/C60A}$ (squares) strains. Thus, although diamide weakly induces the operon (**Fig. 2**, main text) expression of *cst* operon is not likely essential for survival under diamide stress, in contrast to sulfide stress.

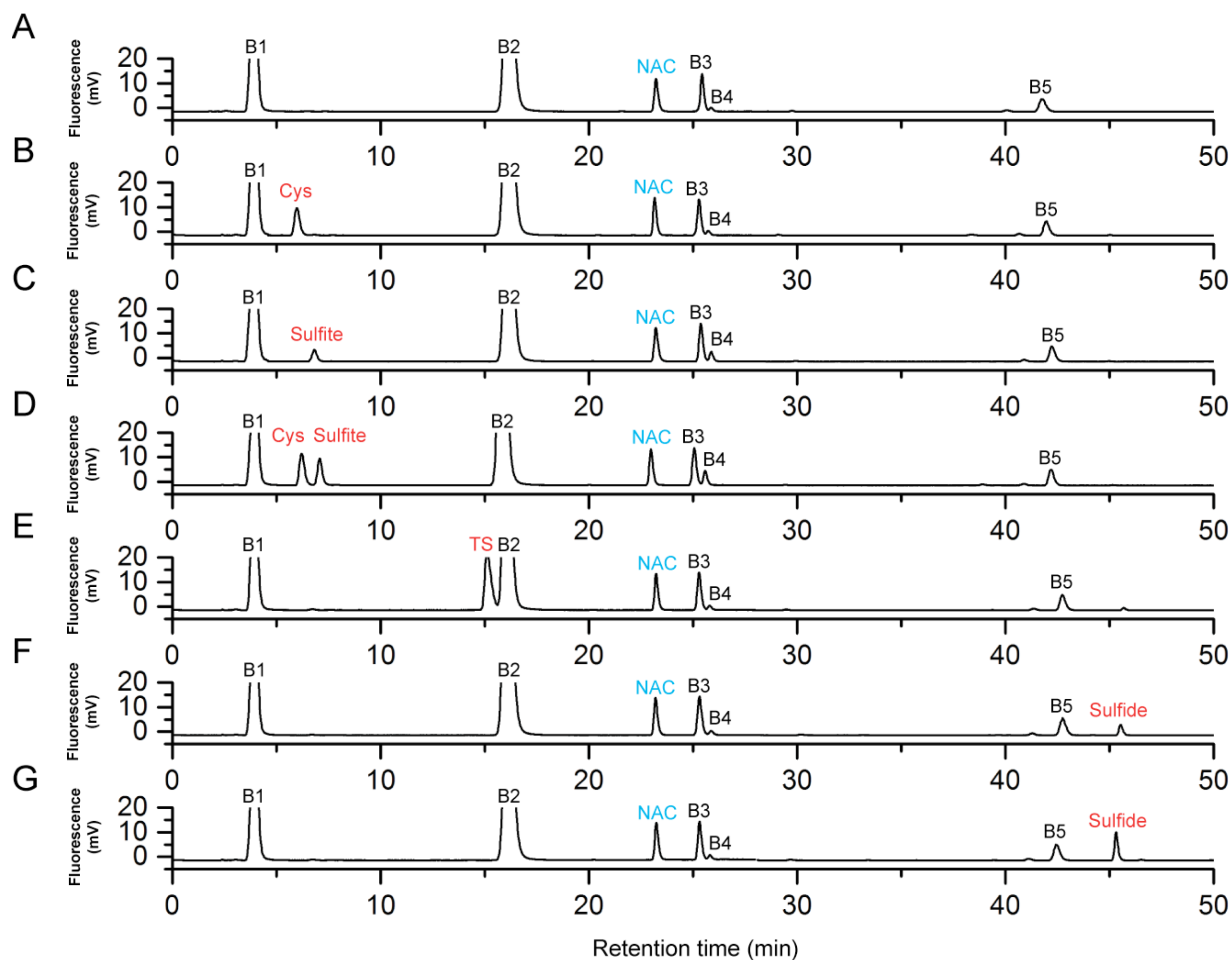

Figure S6. Fluorescence-detected reverse phase HPLC chromatograms of standard compounds used in the identification of major LMW sulfur-containing monobromobimane (mBBr) derivatives (-mB) from *S. aureus* cellular lysates. (**A**) Monobromobimane (mBBr) labeling of buffer components (B, "background") are attributed to the hydrolysis products of monobromobimane (B1 and B2), acetic acid (B3 and B4), and unreacted monobromobimane (B5) as determined by mass spectrometry. (**B**) 20 µM cysteine; (**C**) 20 µM sulfite; (**D**) 20 µM sulfite + 20 µM cysteine; (**E**) 20 µM TS; (**F**) 20 µM NaHS; (**G**) 5 µM Na_2S_4. NAC, *N*-acetyl-*L*-cysteine, used as an internal recovery standard

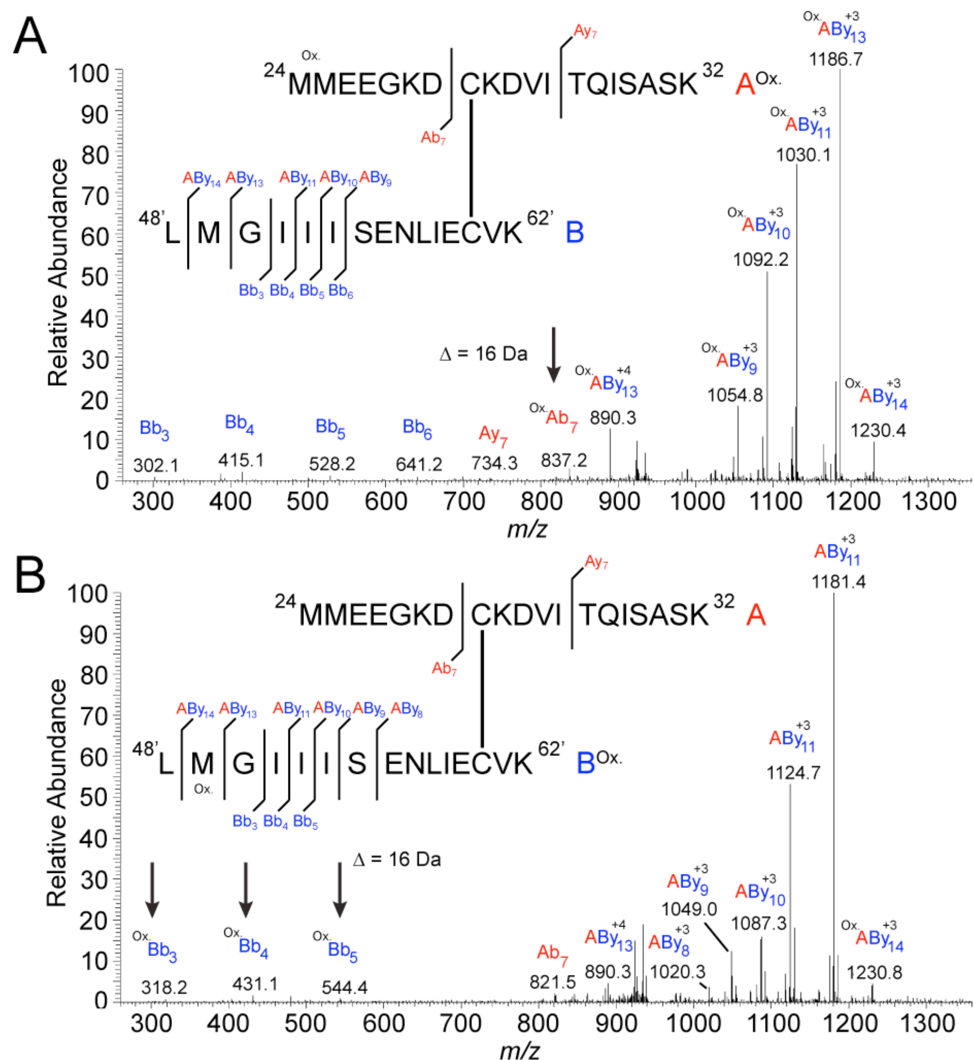

Figure S7. Fragmentation data of sulfide-treated CstR samples can not be attributed to Met oxidation of disulfide-bonded Cys31-Cys60' cross-linked peptides. Cross-linked peptides containing a single Met oxidation event on either the A peptide (**A**) or on the B-peptide (**B**) were identified from an aerobically-digested sample. The parent ions each have a mass of 950.977 Da (950.996 Da expected) in the +4 charge state but are differentiated from sulfur (S) adducted crosslinked peptides by their unique fragmentation pattern. For example, the Ab_7 fragment contains the "A" peptide Met residue with a mass of 821.3 Da and 837.3 Da for the reduced and oxidized forms, respectively. If two oxidations occur on the "A" peptide, the expected mass is 853.3 Da (panel A). An analogous Met oxidation analysis can potentially be found for the "B" peptide with the Bb_{3-6} (panel B). If the parent ion contained a +32 Da shift and the Met residues in the fragmentation spectra display a combined +32 Da or +64 Da mass shift, the shifts would be attributed to Met oxidation and not a trisulfide or tetrasulfide crosslinked Cys31-Cys60' peptide, respectively. These data ensure that the +32 Da and +64 Da shifts observed in so-designated trisulfide and tetrasulfide cross-linked peptides (see **Fig. 4B**, main text) are due to sulfur and not multiple Met oxidation events.

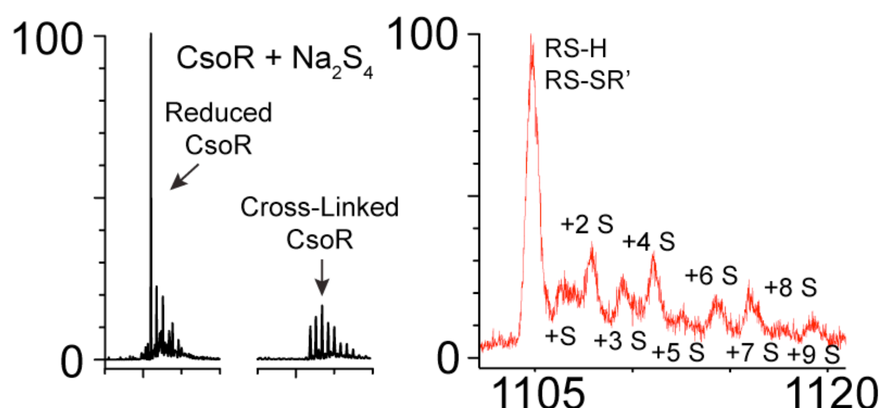

Figure S8. LC-ESI mass spectra of CsoR following reaction with Na_2S_4. The *S. aureus* Cu(I) sensor CsoR was reacted with a 5-fold excess of Na_2S_4 for 17 h and analyzed by LC-ESI-MS. Mass to charge ratios (red) indicate the formation of a series of sulfur cross-linked CsoR protomers with up to nine total sulfur atoms. Deconvoluted spectra (black) indicate the relative abundance of reduced to cross-linked species. In this experiment, it is impossible to determine which sulfurs or how many are involved in cross-linking or as sulfur chains on non-cross-linked cysteines. For a complete mass list, please refer to Table S3.

References

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