Supporting Information

Increased intracellular persulfide levels attenuate HlyU-mediated hemolysin transcriptional activation in *Vibrio cholerae*

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This file contains Supporting Tables S1-S2, Supporting Figures S1-S10.

Table S1: *V. cholerae* strains and primers used for qRT PCR used for this study.

Strain	Description
TND0004	*WT*
TND2438	*ΔlacZ::P_{hlyA}-msfGFP Cm^R*
TND2455	*ΔhlyU::Kan^R, ΔlacZ::P_{hlyA}-msfGFP Cm^R*
TND2459	*ΔhapR::$Spec^R$, ΔlacZ::P_{hlyA}-msfGFP Cm^R*
TND2456	*ΔhlyU::Kan^R, ΔhapR::$Spec^R$, ΔlacZ::P_{hlyA}-msfGFP Cm^R*
TND2533	*Δfur::Tm^R, ΔhapR:$Spec^R$, ΔlacZ::P_{hlyA}-msfGFP Cm^R*
TND2534	*Δfur::Tm^R, ΔhapR::$Spec^R$, ΔhlyU::Kan^R, ΔlacZ::P_{hlyA}-msfGFP Cm^R*
TND2531	*Δfur:Tm^R, ΔlacZ::P_{hlyA}-msfGFP Cm^R*
TND2532	*Δfur:Tm^R, ΔhlyU:Kan^R, ΔlacZ::P_{hlyA}-msfGFP Cm^R*
TND3182	*ΔhapR:$Spec^R$, Δfur::Tm^R, ΔlacZ::P_{hlyA}-msfGFP Cm^R*
TND3183	*ΔhapR:$Spec^R$, Δfur::Tm^R, Δhns::$Carb^R$, ΔlacZ::P_{hlyA}-msfGFP Cm^R*
TND3185	*ΔhapR:$Spec^R$, Δfur::Tm^R, ΔhlyU::Kan^R, ΔlacZ::P_{hlyA}-msfGFP Cm^R*
TND3186	*ΔhapR:$Spec^R$, Δfur::Tm^R, ΔhlyU::Kan^R, Δhns::$Carb^R$, ΔlacZ::P_{hlyA}-msfGFP Cm^R*
Primers PCR	**Sequence**
recA – rev	GCGCAGCAATCTTGTTCTTC
recA – fwd	CGTTTGGATATTCGCCGTACT
hlyA – rev	CTC TGT GGC TGA GGC TTT AT
hlyA – fwd	CGA TGC TTT GTG GGT GAA TAC
GFP – rev	GCTCTTGCACGTATCCTTCT
GFP – fwd	TTGTGACGACTCTGACTTATGG
hlyU – rev	CCA CGC TAG ATG TTG AGA AAG A
hlyU – fwd	GGA CAA TGA ACT GTC GGT AGG

Table S2: Characterized ArsRs in the SSN.

Protein Name*	Organism	Uniprot ID	Cluster number in Network
ecArsR (116)	*Escherichia coli*	P37309	1A
ArsR2 (117)	*Escherichia coli*	A0A142BMN6	1A
ArsR(118)	*Staphylococcus aureus*	P30338	1A
ArsR1&2 (119)	*Geobacillus kaustophilus*	Q5KUX7	1A
ArsR (120)	*Pseudomonas putida*	Q88LK1	1A
AseR(67)	*Bacilus subtilis*	P96677	1A
ArsR (77)	*Corynebacterium glutamicum*	A0A5H1ZR36	1A
Rv2642(105)	*Mycobacterium tuberculosis*	P71941	1A
AztR(121)	*Cyanobacterium anabaena*	Q8ZS91	1B
BxmR(122)	Oscillatoria brevis	Q76L30	1B
NmtR(104)	*Mycobacterium tuberculosis*	O69711	1B
ZiaR(123)	*Synechocystis sp.*	Q55940	1B
CadC(124)	*Staphylococcus aureus*	P20047	1B
SmtB(125)	*Synechococcus elongatus*	P30340	1B
CzrA(126)	*Staphyloccocus aureus*	O85142	1B
CzrA(67)	*Bacilus subtilis*	O31844	1B
CadC(127)	*Listeria innocua serovar 6*	P0A4U2	1B

SmtB(102)	*Thermus thermophilus*	Q72KG0	1B
CadC (127)	*Lysteria monocytogenes*	Q56405	1B
Rv2034(72)	*Mycobacterium tuberculosis*	O53478	2
SdpR (128)	*Bacilus subtilis*	O32242	2
Rv0081(78)	*Mycobacterium tuberculosis*	P9WMI7	3
AntR(129)	*Comamonas testosteroni*	A0A096FLR2	3
BigR(48)	*Acinetobacter baumanni*	D0C7U0	4
YgaV(130)	*Escherichia coli*	P77295	4
NolR(71)	*Rhizobium fredii*	Q83TD2	4
SqrR(50)	*Rhodobacter capsulatus*	D5AT91	4
HlyU(11)**	*Vibrio cholerae serotype O1*	P52695	4
HlyU(20)	*Vibrio parahaemolyticus*	Q87S95	4
HlyU(19)	*Vibrio vulnificus*	A0A3Q0L222	4
BigR(40)	*Xylella fastidiosa*	Q9PFB1	4
BigR(131)	*Agrobacterium tumefaciens*	Q8UAA8	4
PigS (99)	*Serratia* sp. strain ATCC 39006	E7BBJ0	4
SoxR(132)	*Pseudaminobacter salicylatoxidans*	Q5ZQN5	4
ArsR(77)	*Acidithiobacillus ferrooxidans*	B7J952	5
ArsR(133)	*Agrobacterium tumefaciens ArsR 1*	H0HHH0	5
CyeR(134)	*Corynebacterium glutamicum*	A4QI86	6
YczG(135)	*Bacilus subtilis*	O31480	6
RexT(61)	Nostoc sp.	Q8YVV6	6
MerR(136)	*Streptomyces lividans*	P30346	8
PyeR(137)	*Pseudomonas aeruginosa*	Q9HW47	9
KmtR(103)	*Mycobacterium tuberculosis*	O53838	10
PagR(138)	*Bacillus anthracis*	O31178	14
SmtB(139)	*Mycobacterium tuberculosis*	P9WMI4	16
SrnR(73)	*Streptomyces griseus*	Q8L1Y3	21
CmtR(75)	*Streptomyces coelicolor*	Q9RD34	22
CmtR(140)	*Mycobacterium tuberculosis*	P9WMI8	22

* The protein name is followed by the most updated publication with the available biochemistry information. ** This work.

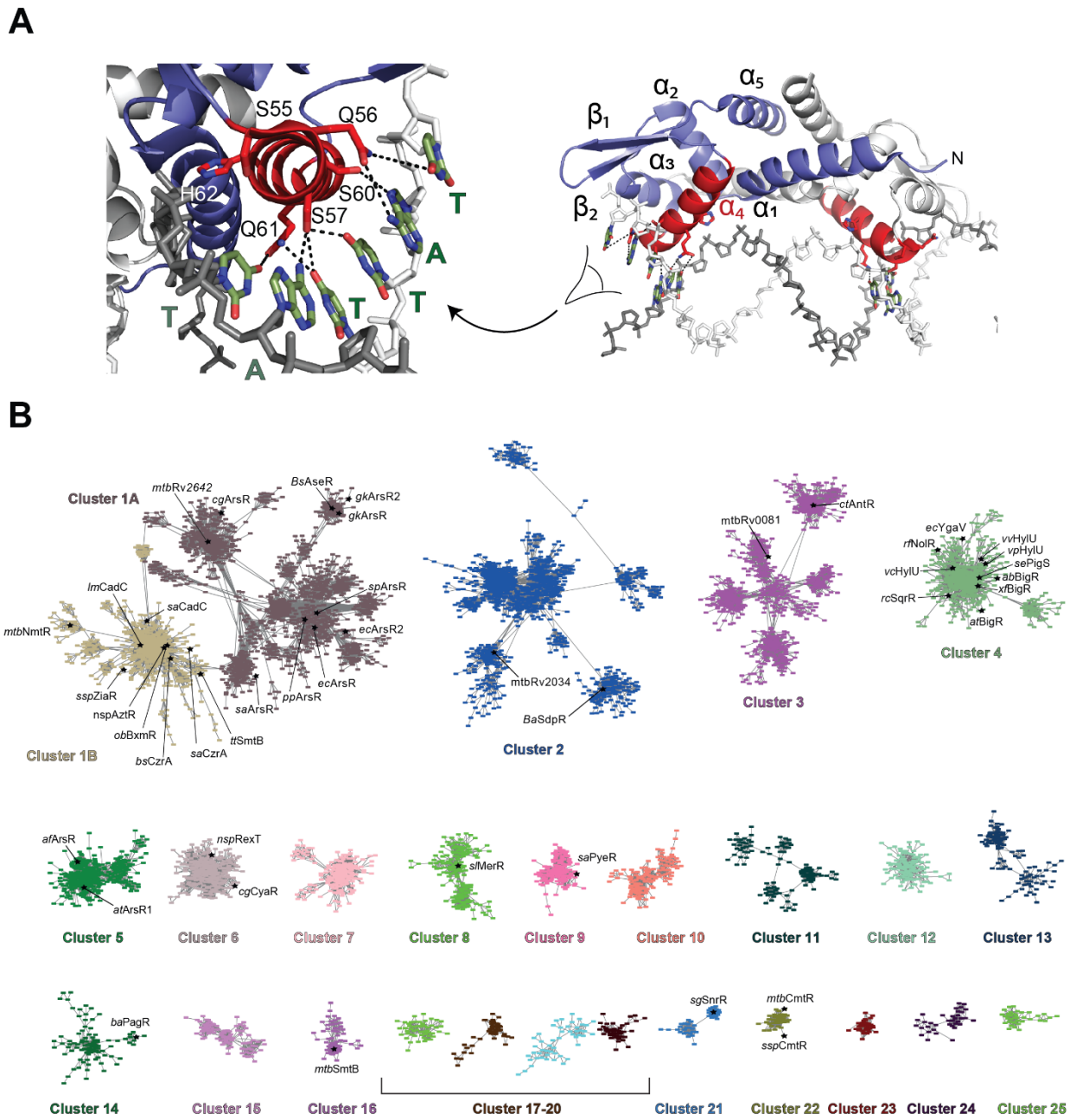

Figure S1. (A) ArsR superfamily proteins architecture (a1-a2-a3-a4-b1-b2-a5) and DNA recognition exemplified by the structure of DNA-bound NolR. The contacts between the protein sequence in a4 (*red*) and bases from DNA (*green*) in the first half-site are marked with *black* dashes. **(B)** All the main clusters generated in the sequence similarity network obtained using Pfam PF01022 and Interpro IPR001845 datasets of annotated ArsRs proteins.

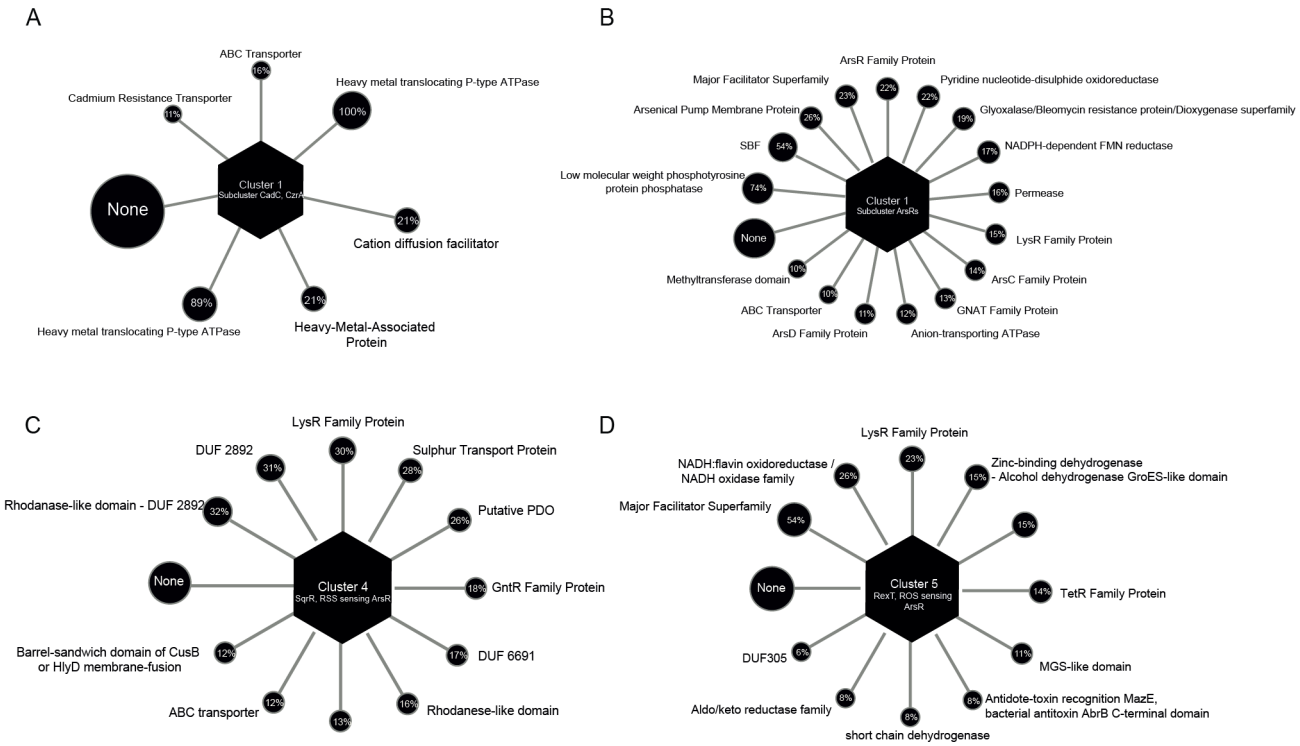

Figure S2. Genomic neighborhood and location of ArsR genes from cluster 1 (A and B), cluster 4 (C) and cluster 5 (D).

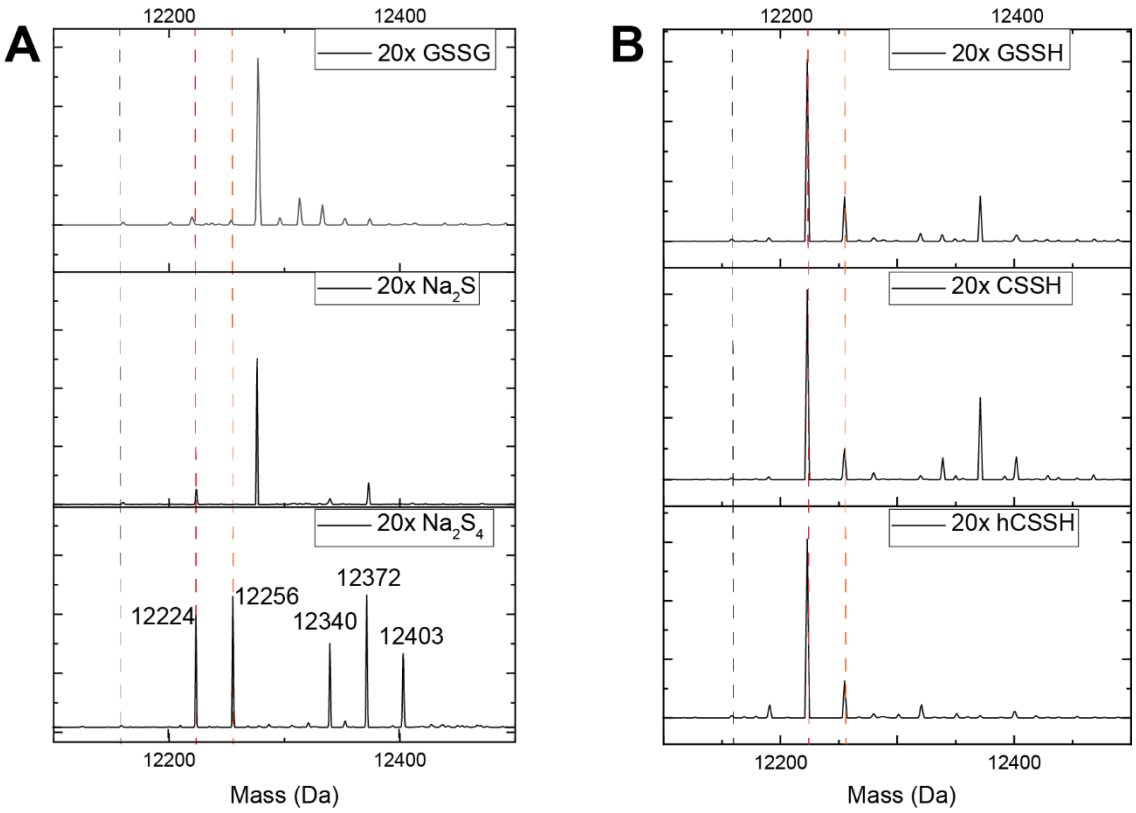

Figure S3. LC-ESI-MS analysis of HlyU *in vitro* reactivity upon a one-hour incubation with a 20-fold excess of **(A)** GSSG, Na_2S and Na_2S_4 and **(B)** organic persulfides GSSH, cysteine and homocysteine persulfides (CSSH and hCSSH, respectively) and then capped with IAM. *Grey* dashed lines correspond to the reduced and uncapped HlyU monomer, while the two *red* dashed lines correspond to the tetrasulfide and pentasulfide species.

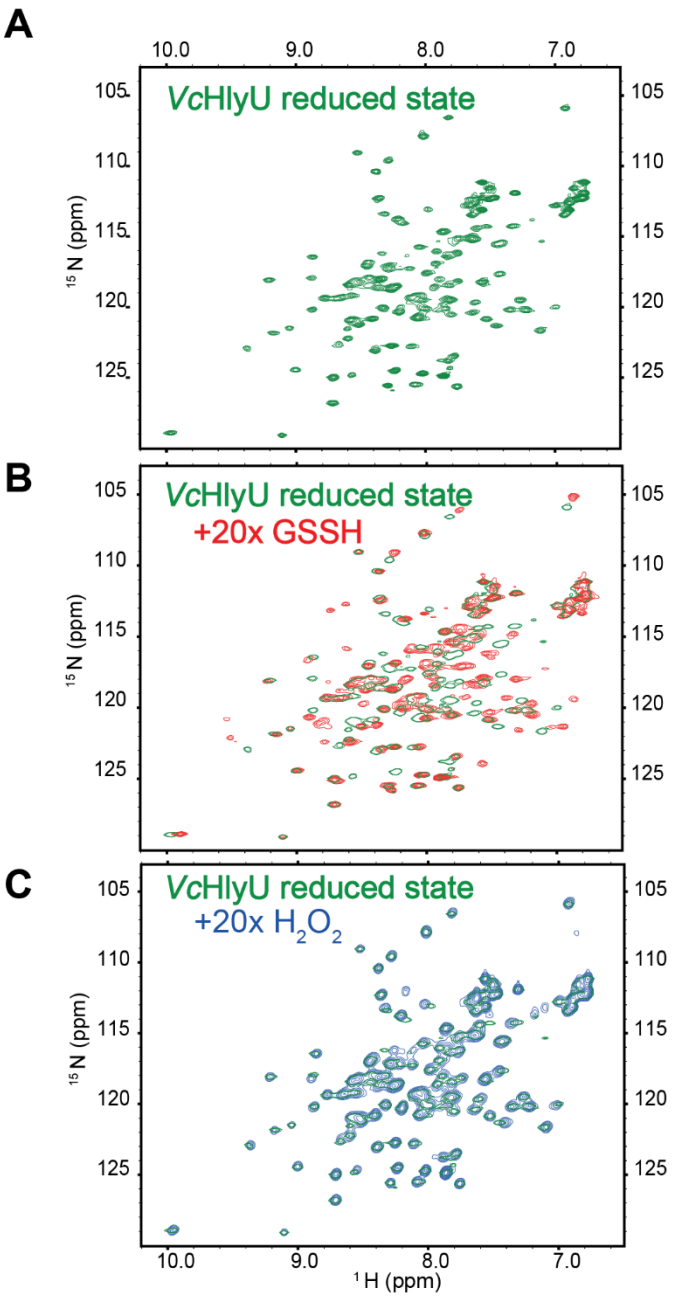

Figure S4. (A) ^{1}H, ^{15}N HSQC spectrum of reduced *Vibrio cholerae* HlyU. **(B)** Overlay of ^{1}H, ^{15}N HSQC spectrum of reduced *Vibrio cholerae* HlyU (*green*) and pre-treated with 20x GSSH (*red*). **(C)** Overlay of ^{1}H, ^{15}N HSQC spectrum of reduced *Vibrio cholerae* HlyU (*green*) and pre-treated with 20x H_2O_2 (*blue*). All spectra were measured at 30 ºC using a 20mM MES pH 6, 250mM NaCl, 1mM EDTA buffer, with the addition of 2mM TCEP in the case of the reduced state.

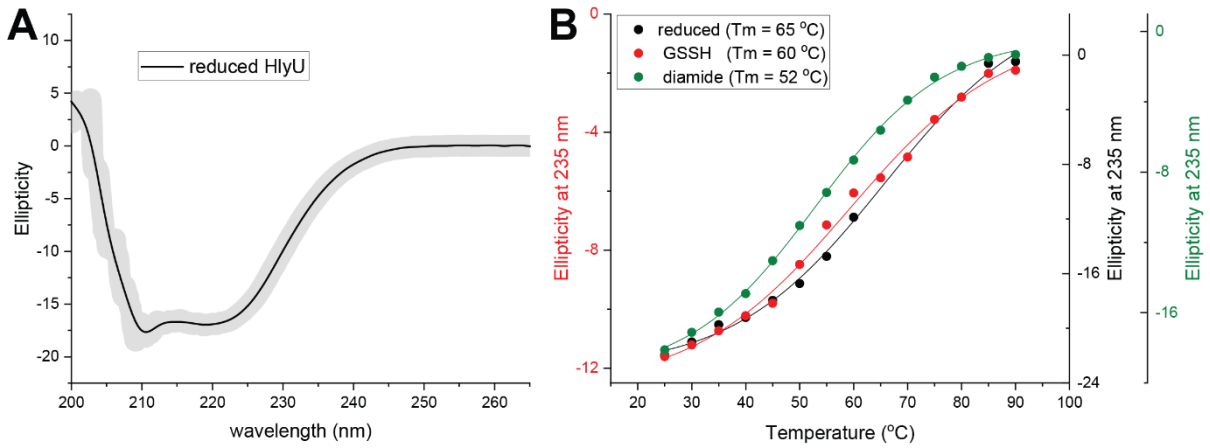

Figure S5. (A) Far-UV CD spectra of reduced HlyU. **(B)** Temperature-induced conformational transitions observed as changes in ellipticity at 232 nm in the 25–90 ºC temperature range for reduced (*black*), tetrasulfide (*red*) and diamide treated (disulfide, *green*) crosslinked HlyU. The line indicates a sigmoidal fitting used to obtain the melting temperature. All spectra were measured using in 25 mM HEPES, pH 7.0, 200 mM NaCl, 1 mM EDTA, with the addition of 1 mM TCEP in the case of the reduced state.

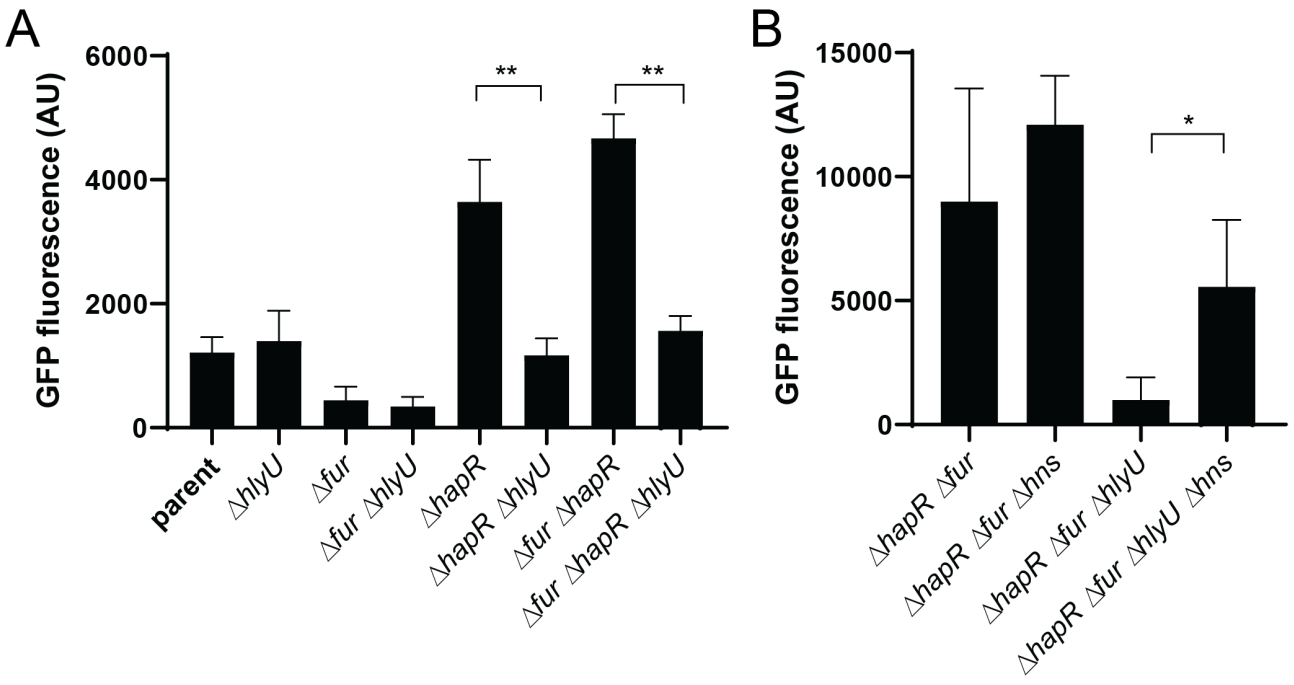

Figure S6. Assessing HlyU activity *in vivo* using a P_{hlyA}-GFP transcriptional reporter. Fluorescence of the indicated *V. cholerae* strains was measured to assess the impact of **(A)** HapR / Fur, and **(B)** HNS on HlyU-dependent activation of P_{hlyA}. Data are from four independent biological replicates and shown as the mean ± SD. Statistical significance was established using a unpaired parametric *t*-test (**p<0.01, *p<0.05).

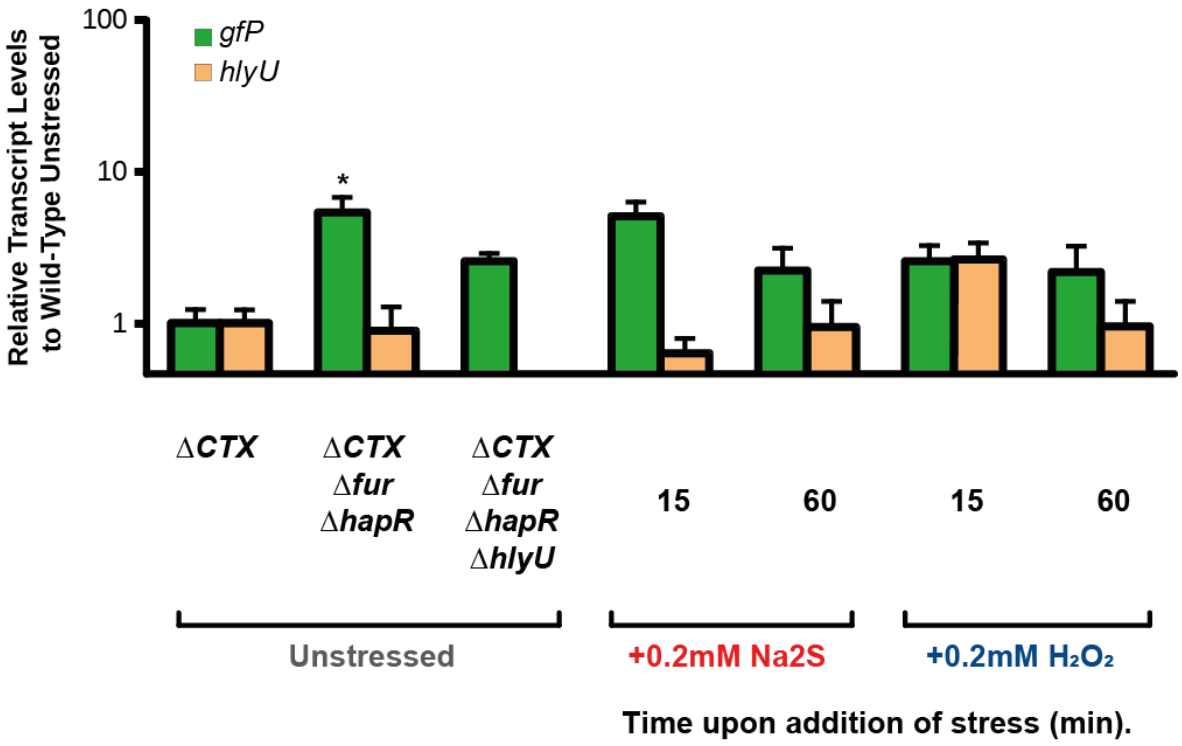

Figure S7. Fold changes transcripts levels of *Vc hlyU* and *gfP* followed by quantitative RT-PCR performed over a ΔCTX Δfur ΔhapR *V. cholerae* strain with the addition of Na_2S or H_2O_2. Transcript values were normalized relative to the transcription level of *recA*. The values correspond to transcript levels relative to wild-type unstressed (WT UN) and are shown as mean ± SD from replicate cultures. Statistical significance was stablished using a paired t test relative to WT UN under the same conditions (**p<0.01, *p<0.05).

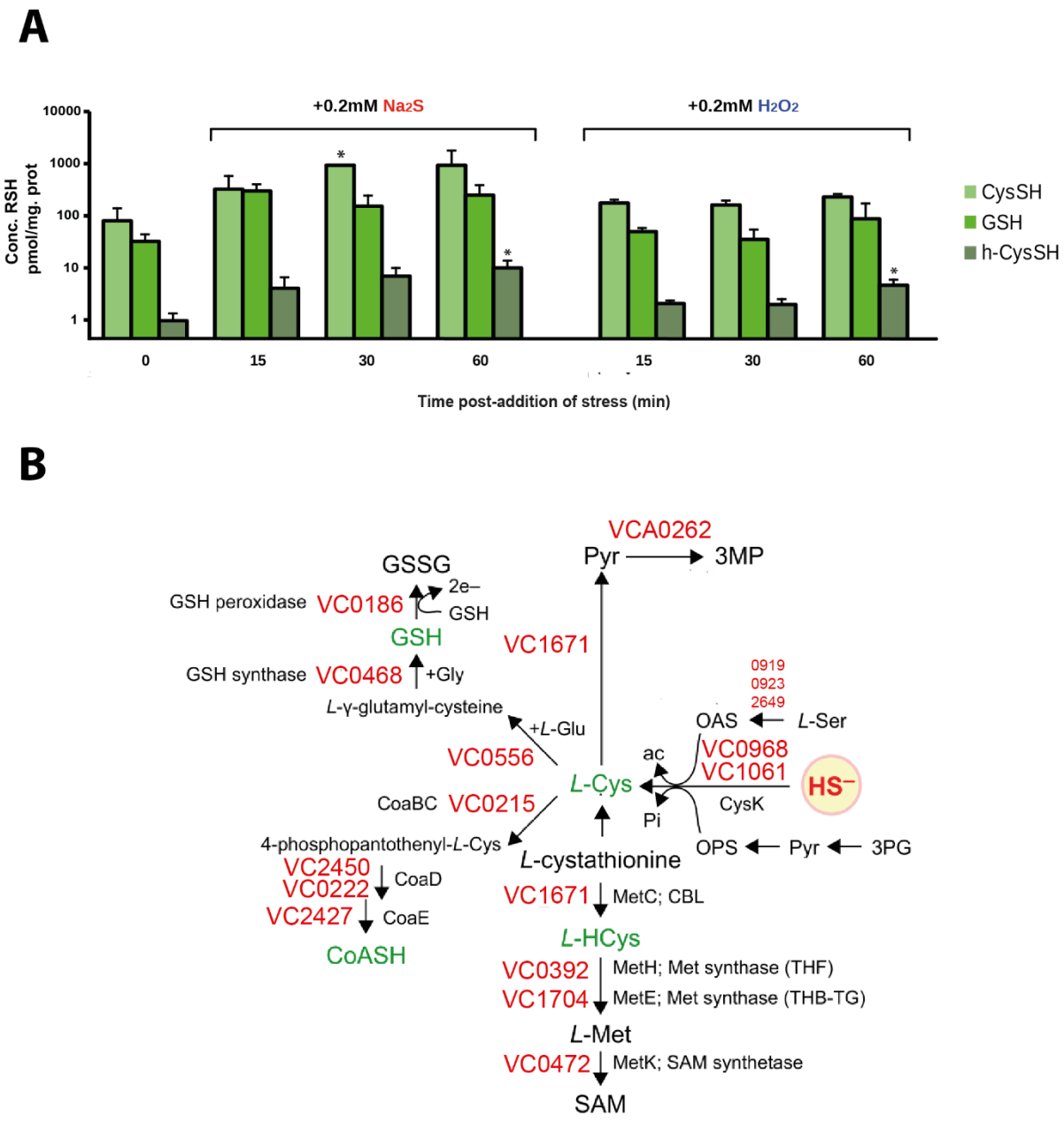

Figure S8. (A) Endogenous concentrations of LMW thiols before and after the addition of Na_2S and H_2O_2 to mid-log-phase cultures (*, $P < 0.05$ using a paired t test relative to WT UN under the same conditions) determined using HPEIAM as capping agent. **(B)** *V. cholerae* genes encoding proteins associated with the biosynthesis of LMW thiols. OPS, O-phospho-L-serine; OAS, O-acetyl-L-serine; ac, acetate; pyr, pyruvate; CBL, cystathonine-γ-lyase. Adapted from reference(48).

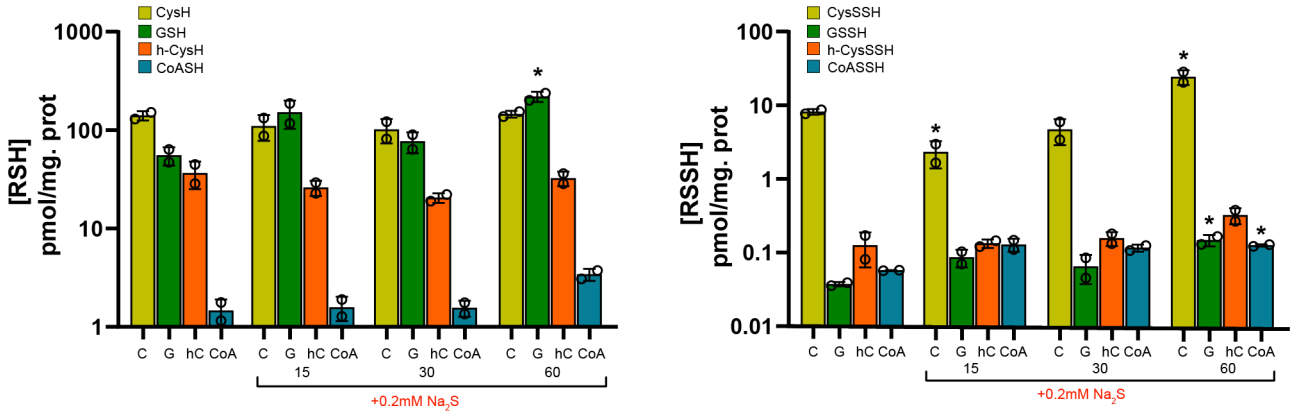

Figure S9. (A) Endogenous concentrations of LMW thiols before and after the addition of 0.2 mM Na_2S to mid-log-phase cultures (*, $p < 0.05$) determined using mBBr as capping agent. **(B)** Endogenous concentrations of LMW persulfides before and after the addition of Na_2S to mid-log-phase cultures (*, $p < 0.05$ using a paired t test relative to WT UN under the same conditions) determined using mBBr as capping agent.

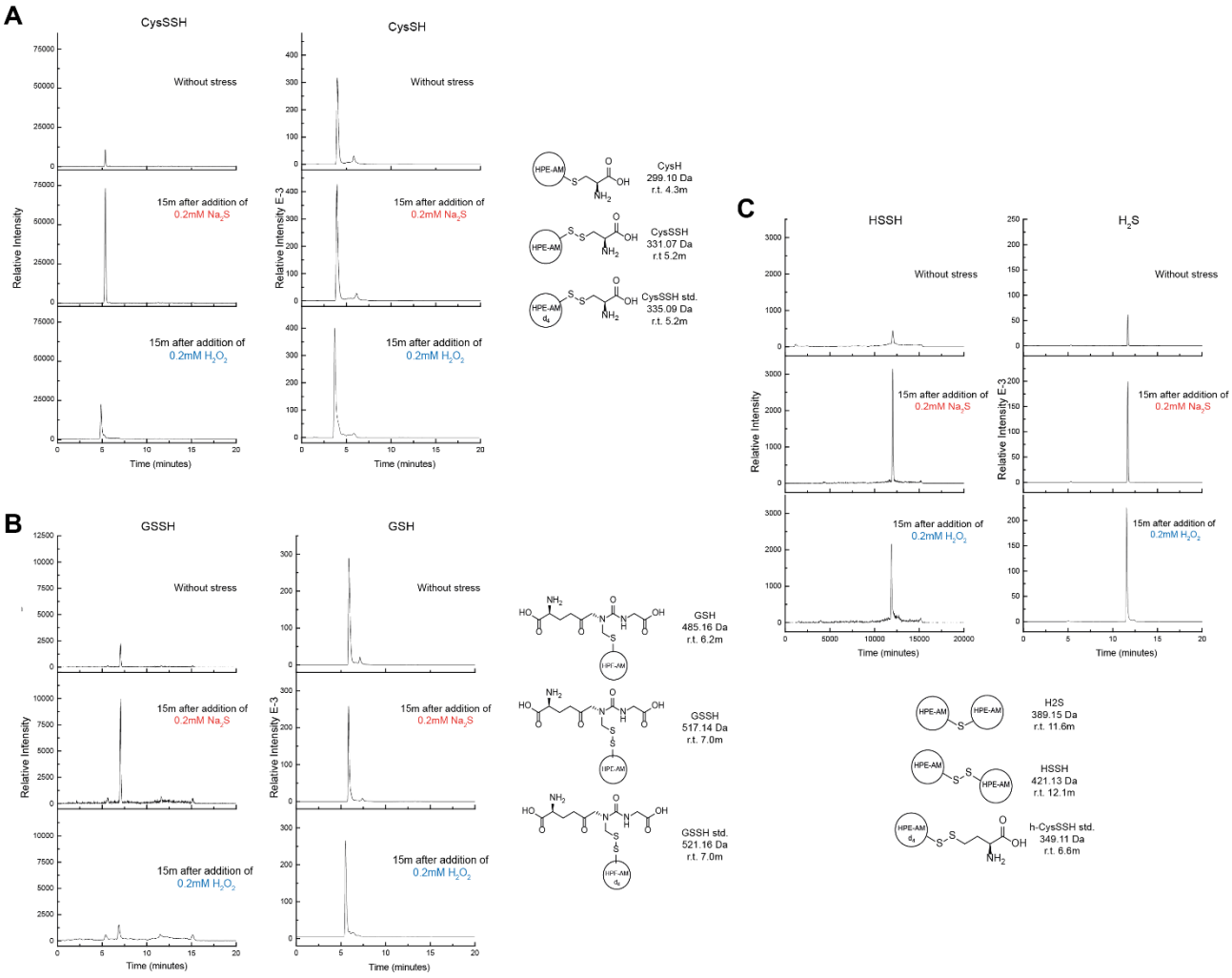

Figure S10. (A) Extracted ion chromatograms corresponding to cysteine thiol and cysteine persulfide (top panel) in unstressed cells and following 15 min of applied stress (Na_2S, middle panel and H_2O_2, bottom panel). **(B)** Extracted ion chromatograms corresponding to glutathione thiol and glutathione persulfide. **(C)** Extracted ion chromatograms corresponding to inorganic sulfide and disulfide. The peaks show in each case the relative intensity of the HPE-IAM labelled metabolites, relative to the intensity of the internal standard. On the right, we show the molecular structure, molecular weight and retention time of the metabolites analyzed in the panels, together with the internal standard used in each case.