Sulfide Homeostasis and Nitroxyl Intersect via Formation of Reactive Sulfur Species in Staphylococcus aureus

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ABSTRACT Staphylococcus aureus is a commensal human pathogen and a major cause of nosocomial infections. As gaseous signaling molecules, endogenous hydrogen sulfide (H₂S) and nitric oxide (NO·) protect S. aureus from antibiotic stress synergistically, which we propose involves the intermediacy of nitroxyl (HNO). Here, we examine the effect of exogenous sulfide and HNO on the transcriptome and the formation of low-molecular-weight (LMW) thiol persulfides of bacillithiol, cysteine, and coenzyme A as representative of reactive sulfur species (RSS) in wild-type and ΔcstR strains of S. aureus. CstR is a per- and polysulfide sensor that controls the expression of a sulfide oxidation and detoxification system. As anticipated, exogenous sulfide induces the cst operon but also indirectly represses much of the CymR regulon which controls cysteine metabolism. A zinc limitation response is also observed, linking sulfide homeostasis to zinc bioavailability. Cellular RSS levels impact the expression of a number of virulence factors, including the exotoxins, particularly apparent in the ΔcstR strain. HNO, like sulfide, induces the cst operon but also indirectly represses much of the CymR regulon which controls cysteine metabolism. A zinc limitation response is also observed, linking sulfide homeostasis to zinc bioavailability. Cellular RSS levels impact the expression of a number of virulence factors, including the exotoxins, particularly apparent in the ΔcstR strain. HNO, like sulfide, induces the cst operon as well as other genes regulated by exogenous sulfide, a finding that is traced to a direct reaction of CstR with HNO and to an endogenous perturbation in cellular RSS, possibly originating from disassembly of Fe-S clusters. More broadly, HNO induces a transcriptomic response to Fe overload, Cu toxicity, and reactive oxygen and reactive nitrogen species and shares similarity with the sigB regulon. This work reveals an H₂S/NO· interplay in S. aureus that impacts transition metal homeostasis and virulence gene expression.

IMPORTANCE Hydrogen sulfide (H₂S) is a toxic molecule and a recently described gasotransmitter in vertebrates whose function in bacteria is not well understood. In this work, we describe the transcriptomic response of the major human pathogen Staphylococcus aureus to quantified changes in levels of cellular organic reactive sulfur species, which are effector molecules involved in H₂S signaling. We show that nitroxyl (HNO), a recently described signaling intermediate proposed to originate from the interplay of H₂S and nitric oxide, also induces changes in cellular sulfur speciation and transition metal homeostasis, thus linking sulfide homeostasis to an adaptive response to antimicrobial reactive nitrogen species.

KEYWORDS hydrogen sulfide, nitric oxide, nitroxyl, persulfide, reactive nitrogen species, reactive sulfur species, transcriptomics

Hydrogen sulfide (H₂S) is both a toxic gas and a substrate for the biosynthesis of cysteine, an essential amino acid required for the synthesis of low-molecular-weight (LMW) thiols and the biogenesis of iron-sulfur (Fe-S) proteins (1, 2). Cysteine and
H₂S are also metabolic precursors for methionine and other sulfur-containing enzyme cofactors and thus significantly impact a wide range of metabolic activities in the cell (Fig. 1A). In bacteria, including the human pathogen *Staphylococcus aureus*, the cysteine pool is controlled by the global regulator CymR, which impacts sulfur metabolism, virulence gene expression, and survival of the organism both inside and outside the host (3–5). Sulfide (the term which we use here to refer collectively to H₂S, HS⁻, and S²⁻) can be assimilated from exogenous sulfur sources or produced endogenously primarily by enzymes of the transsulfuration pathway, including cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) (Fig. 1A). The toxicity of H₂S coupled with its beneficial functions suggests a need for sulfide homeostasis, in which the cellular concentrations of sulfide and sulfide-derived reactive sulfur species are tightly regulated.

H₂S freely passes through cell membranes, unlike its deprotonated conjugate bases (HS⁻ and S²⁻), and this property underscores recent suggestions that H₂S, like nitric oxide (NO) and carbon monoxide (CO), is a vertebrate gasotransmitter that functions as an endothelium-derived vasorelaxer (6, 7). Recent findings in mammalian cells suggest that H₂S and NO functionally interact to form nitroxyl (HNO), the one-electron reduced and protonated form of NO, which controls the activity of the sensory chemoreceptor channel TRPA1 (8–10) (Fig. 1B). Likewise, in bacterial systems, H₂S (11) and NO (12) are reported to act synergistically to protect a number of bacterial strains against antibiotic stress, a finding that originates with the bacterially encoded nitric oxide synthases which are generally protective against immune oxidative attack (13, 14). In vitro experiments firmly establish that this cross talk between H₂S and NO leads to the formation of HNO as a primary species, as well as of thiol persulfides (RSS⁻) and organic (RSSₙ⁻) and inorganic (HS⁻) polysulfides (n > 1) as bioactive products (Fig. 1B) (15). These per- and polysulfide species, collectively termed reactive sulfur species (RSS), are reportedly maintained in relatively high concentrations (0.01 to 0.1 mM) in mammalian cells and are proposed to function as true small-molecule signaling species in an H₂S signaling pathway (16).

Although our understanding of the biological impact of HNO is not yet complete, HNO appears to be characterized by a chemical reactivity and biogenesis profile that is distinct from that of NO. It is thought that the reduction of NO by specific Fe(II)-heme and high-spin Fe(II) or Mn(II) complexes makes them potential sources of endogenous HNO in living cells (17). HNO is a highly reactive and short-lived molecule that self-quenches via dimer formation to yield nitrous oxide (N₂O) (18). However, prior to self-quenching, this potent electrophile is capable of reacting with nucleophilic thiolate groups to form sulfonamides (Fig. 1C) (19, 20). In addition, while HNO reacts only slowly with molecular oxygen at neutral pH to generate peroxynitrite (ONOO⁻) (21), ONOO⁻ is formed from NO⁻ and superoxide anion at diffusion-controlled rates (Fig. 1C) (22, 23). Thus, the presence of multiple reactive nitrogen species (RNS) and reactive oxygen species (ROS) at the host-pathogen interface may lead to the formation of ONOO⁻, which is a strong oxidant that causes lipid peroxidation, nitration of aromatic residues, thiol oxidation, and disassembly of Fe-S clusters (24) (Fig. 1C).

There is emerging evidence that endogenously produced sulfide and a pool of RSS and perhaps downstream products (Fig. 1B) protect cells against the effects of ROS (16, 25, 26). Indeed, proteome S-sulfhydration might constitute a reservoir of persulfidated cysteines while driving up- and downregulation of metabolic pathways to provide protection against oxidative stress (16, 25, 27). In this model, cells must be capable of both biosynthesis and clearance of RSS to maintain sulfide homeostasis. In previous work, we discovered and characterized a novel per- and polysulfide-sensing dithiol-containing repressor from the major nosocomial pathogen *S. aureus*, named CstR (for “CsoR-like sulfurtransferase repressor”) (28, 29). CstR represses transcription of *cstA*, *cstB*, and *sgr*, which together encode an H₂S oxidation system (S²⁻ to thiosulfate, S₂O₃²⁻) (26, 30, 31). Sulfide-responsive and RSS-sensing repressors that are functionally analogous to CstR and yet are structurally unrelated are also found and have recently been
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FIG 1 Sulfide (S\(_2^–\)) homeostasis and small-molecule chemistry that couples reactive sulfur species (RSS) and reactive nitrogen species (RNS). (A) Sulfide (S\(_2^–\), H\(_2\)S, and H\(_3\)S) and O-acetyl-L-serine (OAS) are substrates for cysteine synthase (CysK) to form L-cysteine. L-Cysteine is the biosynthetic precursor to the major cellular reducing thiol in S. aureus, bacillithiol (BSH), and to coenzyme A (CoASH), which plays important roles in acyl-transfer reactions. L-Cysteine is also the precursor to other major sulfur-containing cofactors, including [Fe-S] clusters, biotin, lipoic acid, and thiamine pyrophosphate (TPP). Lipoic acid and TPP function in the pyruvate dehydrogenase (pyruvate deHase) complex (upper right), used to synthesize acetyl-CoA, which is fed into the tricarboxylic acid (TCA) cycle and other cellular processes. S\(_2^–\) can be accumulate in cells either from exogenous sulfide sources or endogenously via the activity of two enzymes in a transsulfuration pathway, cystathionine \(\gamma\)-synthase (CBS) and cystathionine \(\gamma\)-lyase (CSE), which converts homocysteine to L-cysteine. (B) Nitroxyl (HNO) defines an intersection of sulfide, LMW thiols (RSH), reactive sulfur species (RSS), reactive oxygen species (ox), and nitric oxide (NO). Sulfide can lead to the accumulation of inorganic polysulfides (HS\(_n\)H) and of organic persulfides (RSS\(_n\)H), collectively termed reactive sulfur species (RSS), for which there is evidence in mammalian cells (16) and bacterial cells (26). These RSS are sensed by CsrR (29), leading to the upregulation of a sulfide oxidation system that includes a canonical sulfide:quinone oxidoreductase (SQR) (26, 81). The RSS can also be derived from exogenous or endogenous NO, which reacts with oxidized sulfur species (S\(^{m+n}\), RS\(^{m+n}\)) to create the nitrosotrichnionic acid (HSNO) and organic nitrosothiol (RSNO), which in turn react with S\(_2^–\) to make HNO (82-86). HNO can also be made (Continued on next page)
characterized in Gram-negative bacteria, suggesting that sulfide homeostasis may be more widespread than previously anticipated (32, 33).

In this work, we employed transcriptomic and organic persulfide metabolite profiling approaches to investigate the cellular response of *S. aureus* to sodium sulfide and a commonly used HNO donor, Angeli’s salt (AS) (34) (Fig. 1C), added exogenously to cells. We found that sulfide treatment induces the *cst* operon as expected (29) and strongly downregulates a subset of genes regulated by CymR, the master regulator of cysteine biosynthesis (3, 5). We further show that AS treatment bears some similarity to sulfide stress, a finding directly attributable to HNO and not nitrite (NO₂⁻) (29). Both sulfide stress and HNO also result in a significant zinc starvation response, while the ΔcstR strain, characterized by lower levels of RSS, shows significant repression of the expression of immunomodulators and superantigen toxins, some of which are controlled by the global virulence regulator MgrA (35). The implications of these findings for the biological pathway(s) influenced by sulfide/RSS signaling and H₂S/HNO interplay in virulence gene expression and transition metal bioavailability are discussed.

**RESULTS AND DISCUSSION**

**Transcriptomic analysis of the effects of exogenous sulfide stress and a ΔcstR strain.** Previous work showed that addition of sodium sulfide to *S. aureus* cells aerobically grown to the early log phase resulted in the transient induction of the *cst* operon and massive accumulation of organic LMW persulfides in cells (26, 29). In contrast, unregulated expression of genes in a *cst* operon in a ΔcstR strain reduced the levels of these cellular RSS below those seen with wild-type cells (see below). In order to understand the impact of cellular sulfide and RSS concentrations on global gene expression, we carried out a transcriptomic analysis of mid-log *S. aureus* strain Newman treated with 0.2 mM NaHS for 10 min versus an untreated ΔcstR strain and of both relative to untreated wild-type cells (see Table S1A and Fig. S1 in the supplemental material). Totals of 38 genes and 37 genes, respectively, exhibited an increase or decrease in expression of more than 3.0-fold (1 standard deviation from the mean induction level; see Table S1A) in sulfide-treated cells and in untreated wild-type cells (Tables 1 and 2, respectively). These genes included *cstA*, *cstB*, and *sqr*, all direct targets of CstR regulation (29), as well as a number of enzymes and regulators involved in sugar (*glpF, marR, gapB, scrR, gntK, and gntR*) and amino acid (*putA*) metabolism.

A subset of genes that were upregulated in both the sulfide-stressed wild-type and ΔcstR cultures (Table 3) relative to untreated wild-type *S. aureus* cultures included *aldA* (NWMN_0113), encoding an uncharacterized aldehyde dehydrogenase, and hypothetical operons beginning with NWMN_0134 (NWMN_0134 to NWMN_0137) and NWMN_0151 (NWMN_0151 to NWMN_0154), the latter of which is associated with changes in carbohydrate metabolism and uptake in other *Staphylococcus* strains. Although some of these sulfide-inducible genes are also induced in the ΔcstR strain, none would appear to be direct targets of CstR regulation since they lack clearly identifiable CstR operators upstream (28) in the immediately adjacent intergenic regions, a finding supported by our clustering analysis (Fig. 2A, bottom). These data, taken collectively, suggest that CstR likely directly regulates the expression of a single operon, *cst*, with other genes induced by sulfide treatment likely indirectly influenced by metabolic changes in the cell, which includes RSS.

Comparing this list of sulfide-induced genes relative to the untreated control wild-type strain with results of other transcriptomics experiments using the *S. aureus*
transcript regulatory network analysis tool (SATRAT) (36) reveals that a majority of these genes were oppositely (down)regulated by oxidative stress induced by short-time-scale exogenously added hydrogen peroxide (Fig. S2A and C) (37) and hypochlorous acid. In addition, the ratio of reduced to oxidized bacillithiol (BSH), one of the major LMW thiols in S. aureus (see below), increased upon sulfide treatment relative to untreated cells (Fig. S3), which is opposite what is expected under conditions of oxidative stress. These data collectively reveal that the transcriptomic response induced by changes in cellular RSS is opposite that induced by this potent oxidant (Table S1A). Thus, RSS and ROS transcriptional programs operate independently in the cell, consistent with what is known about the inducer specificity of CstR relative to that of PerR, the major ROS-sensing repressor in S. aureus (29, 38).

Exogenous sulfide induces a zinc limitation transcriptomic response. In addition to the transcriptomic response to exogenous sulfide treatment described above, exogenous sulfide stress also induces a pronounced zinc limitation response in S. aureus that is similar to that mediated by the antimicrobial protein calprotectin (CP) (Fig. 2B and Fig. 3). For example, sulfide treatment induces strong upregulation of the zinc uptake repressor (Zur) regulon (39) (Table 1; Table S1A), while downregulating the expression of czrAB (zntRA) (40), encoding the Zn efflux regulator and cation diffusion facilitator transporter (Table 2) (41, 42). Sulfide treatment also leads to upregulation of mntABC, controlled by the Mn-sensing repressor MntR (43), but no measurable Fe
limitation response, in contrast to CP treatment (Fig. 2B). Genes that are upregulated by both exogenous sulfide treatment and CP treatment and linked to transition metal homeostasis include NWMN_2481, encoding a putative COG0523 G3E family GTPase linked to zinc homeostasis in other organisms (44, 45); two genes encoding uncharacterized pyridine nucleotide disulfide oxidoreductases (NWMN_0815 and NWMN_2370); rpmG2 and rpsN2, encoding two non-zinc-containing paralogs of ribosomal proteins (46); the adcA gene encoding a zinc-binding lipoprotein (NWMN_2306); and the entire cnt gene cluster, cntA to cntF (cntA-F) (Table 1) (Fig. 2B and Fig. 3). cntA-F is reported to encode a Co/Ni uptake system that is expressed upon metal limitation (47), while cntKLM encodes the biosynthetic machinery that produces a broad-spectrum nicotianamine-like metallophore that is capable of scavenging metals from the environment (48).

Induction of a zinc starvation response by application of exogenously added sulfide can be traced to the ability of bisulfide (HS\(^-\)/H\(_2\)S\(_2\)) salts to form stable coordination complexes with transition metals, leading to their precipitation from solution and making them nonbioavailable (49). Analysis of the growth medium before and after addition of 0.2 mM Na\(_2\)S reveals an approximately 10-fold decrease in total Zn levels, with relatively smaller reductions in Cu(II) and Ni and no change in Mn and Fe (Fig. S4A). This suggests that increased levels of endogenous sulfide in the cytoplasm may lead to a reduction in the bioavailability of intracellular transition metals, particularly Zn, by chelation.

**Part of the CymR regulon is repressed by exogenous sulfide treatment.** The suite of genes that are strongly (≥3.0-fold) repressed under conditions of exogenous sulfide stress are largely limited to a subset of CymR-regulated genes (5, 50), defined by differential expression in a cymR mutant S. aureus strain, strain N315 (Table 2; Fig. 4). CymR is the master regulator of cysteine metabolism, repressing the expression of genes that lead to cysteine biosynthesis, and thus controls the response of the organism to a sulfur source. Increased exogenous sulfide levels lead to increased levels of intracellular sulfide (29) and LMW thiol persulfides (Fig. 5C). These sulfide-repressed genes include the operon NWMN_0115 to NWMN_0119, which is thought to encode a taurine (sulfonate) importer (NWMN_0116 to NWMN_0118); an operon encoding an uncharacterized ABC transporter, which is proposed to be involved in glutathione assimilation (NWMN_0143 to NWMN_0146) (3); a gene encoding a cysteine synthase (cysK); and genes encoding two enzymes of the transsulfuration pathway, CBS (encoded by cysM) and CSE (metB), that allow sulfur assimilation from the major human thiol, homocysteine. In addition, the expression levels of genes encoding methionine (NWMN_0246 to NWMN_0428) and cystine (NWMN_0374 and NWMN_1749 to NWMN_1751) ABC transporters and an operon (NWMN_1951 to NWMN_1952) encoding a TusA-like sulfurrtransferase and a putative thiosulfate importer are also repressed.
TABLE 1 List of genes upregulated in response to NaHS stress (≥3-fold) or by Angeli’s salt (≥10-fold)

| Locus tag (Newman) | Locus tag (N315) | Gene name | Fold induction (NaHS stress) (S<sup>2</sup>·<sup>−1</sup>) | Fold induction (Angeli’s salt) | Function | Plus CP treatment<sup>b</sup> (fold increase)? | Δcst<sup>R</sup> regulation? |
|-------------------|----------------|-----------|---------------------------------|-------------------------------|----------|------------------------------------------|--------------------------|
| NWMN_0026 | SA0080 | tauE | 5.5 | Putative sulfonate/thiosulfate (TS) efflux | Yes |
| NWMN_0027 | SA0082 | ctaA | 6.1 | Multidomain sulfotransferase<sup>a</sup> | Yes |
| NWMN_0028 | SA0083 | ctaB | 5.8 | Persulfide dioxygenase-sulfur transferase<sup>b</sup> | Yes |
| NWMN_0029 | SA0084 | sqd | 4.9 | Sulfide-quinone reductase<sup>b</sup> | Yes |
| NWMN_0047 | SA0098 | cstR | 15.0 | Hypothetical | |
| NWMN_0048 | SA0099 | cstA | 6.1 | Multidomain sulfurtransferase | |
| NWMN_0071 | SA0122 | aldA | 2.0<sup>e</sup> | Acetoin dehydrogenase-like | Yes |
| NWMN_0113 | SA0162 | aldA | 4.8 | Aldehyde dehydrogenase-like | Yes |
| NWMN_0134 | SA0184 |  | 4.4 | Hypothetical (NWMN_0134–0137; carbohydrate metabolism) | |
| NWMN_0151 | SA0206 |  | 8.6 | Hypothetical (NWMN_0151–0154 operon) | Yes |
| NWMN_0162 | SA0218 | pflB | 8.1 | Formate acetyltransferase | |
| NWMN_0163 | SA0219 |  | 19.1 | Formate-lyase activating enzyme | |
| NWMN_0173 | SA0229 |  | 2.2 | Hypothetical | |
| NWMN_0174 | SA0230 |  | 2.1 | Hypothetical | |
| NWMN_0175 | SA0231 |  | 2.2 | Flavohemoprotein | Yes (3.4) |
| NWMN_0180 | SA0236 |  | 8.0 | Glyoxalase-like protein | |
| NWMN_0330 | SA0327 |  | 9.2 | NADH-dependent flavin mononucleotide (FMN) reductase (like NWMN_2421) | |
| NWMN_0331 | SA0328 |  | 9.2 | NADH-dependent flavin mononucleotide (FMN) reductase (like NWMN_2421) | |
| NWMN_0365 | SA0365 |  | 5.7<sup>e</sup> | Alkyl hydroperoxide reductase subunit F | |
| NWMN_0366 | SA0366 |  | 5.8<sup>e</sup> | Alkyl hydroperoxide reductase subunit C | |
| NWMN_0367 | SA0367 |  | 13.7 | Nitroreductase family protein | |
| NWMN_0410 | SA0396 | lpi7nm | 4.0 | Tandem lipoprotein (NWMN_0410–0411 operon) | |
| NWMN_0417 | SA0410 | cobW | 7.2 | Putative COG0523 GTPase | |
| NWMN_0418 | SA0411 | ndhF | 3.6 | Putative NADH dehydrogenase subunit 5 | |
| NWMN_0484 | SA0480 | ctsR | 16.3 | Transcriptional regulator CtsR (stress induced) | |
| NWMN_0485 | SA0481 |  | 20.6 | UvB/UvC motif-containing protein | Yes (2.9) |
| NWMN_0486 | SA0482 |  | 16.1 | ATP:guanido phosphotransferase | |
| NWMN_0487 | SA0483 | cipC | 12.7 | ClpC protease, ATP-binding subunit | |
| NWMN_0587 | SA0572 |  | 15.5 | Hypothetical | |
| NWMN_0669 | SA0655 | fruA | 14.2 | Fructose permease (NWMN_0667–0669, frc operon) | |
| NWMN_0815 | SA0806 |  | 17.5 | NADH-dependent pyridine nucleotide disulfide oxidoreductase (short-chain dehydrogenase/reductase [SDR]) | Yes (229) |
| NWMN_0826 | SA0817 |  | 13.1 | NADH-dependent flavin oxidoreductase | |
| NWMN_0845 | SA0835 |  | 28.2 | ClpB protease, ATP binding subunit | |
| NWMN_0900 | SA0900 |  | 3.2 | Hypothetical (57 residues) | |
| NWMN_1084 | SA1084 |  | 3.5 | Anti-protein (44 residues) | |
| NWMN_1207 | SA1140 | glpF | 4.3 | Glycerol uptake facilitator protein | Yes |
| NWMN_1224 | SA1170 |  | 3.4 | Hypothetical (82 residues) | |
| NWMN_1246 | SA1170 | katA | 2.5 | Catalase | |
| NWMN_1247 | SA1170 | rpmG2 | 6.6 | Non-Zn-containing paralog rpmG | Yes (2.0) |
| NWMN_1248 | SA1171 | rpsN2 | 9.1 | Non-Zn-containing paralog rpsN (S14) | Yes (27.3) |
| NWMN_1339 | SA1339 | marR | 6.3 | Putative PurR/LacI family repressor | Yes |
| NWMN_1409 | SA1409 | dnaJ | 9.1 | Molecular chaperone DnaK (hsp70) | |
| NWMN_1410 | SA1410 | grpE | 8.9 | Heat shock protein GrpE | |
| NWMN_1411 | SA1411 | hrcA | 11.1 | Heat-inducible transcriptional repressor | |
| NWMN_1580 | SA1510 | gapB | 3.7 | Glyceraldehyde-3-phosphate dehydrogenase 2 | |
| NWMN_1658 | SA1585 | putA | 5.8 | Proline dehydrogenase | Yes |
| NWMN_1709 | SA1709 | bsaG | 15.2 | ABC transporter protein: lactobionic | |
| NWMN_1710 | SA1710 | bsaE | 9.5 | ABC transporter protein: lactobionic | |
| NWMN_1711 | SA1711 | bsaF | 8.6 | ABC transporter protein: lactobionic | |
| NWMN_1929 | SA1929 | dapE | 9.5 | Succinyl-diaminopimelate desuccinylase (dinuclear) | |
| NWMN_1949 | SA1947 | scrS | 3.1 | Succinate operon repressor | |
| NWMN_2026 | SA1924 |  | 11.3 | Aldehyde dehydrogenase family | |
| NWMN_2043 | SA1941 |  | 25.8 | Dps; monoheme Fe-containing ferritin | Yes (3.0) |
| NWMN_2044 | SA1942 |  | 8.0 | Hypothetical; predicted disulfide oxidoreductase | Yes (2.9) |
| NWMN_2048 | SA1946 |  | 19.1 | Hypothetical | |
| NWMN_2059 | SA1962 | mltA | 14.7 | Mannitol-specific IIA component (2057–2060 operon) | |
| NWMN_2060 | SA1963 | mltD | 15.4 | Mannitol-1-phosphate 5-dehydrogenase | |

(Continued on next page)
(3). Loss of cymR in a cymR mutant results in overexpression of the CymR regulon, which
is a transcriptomic response that is opposite the repression of the CymR regulon that we observed with exogenous sulfide. Two other genes that are strongly repressed and not part of the CymR regulon are NWMN_0557 and NWMN_0558, encoding a putative flavin adenine dinucleotide (FAD)-dependent oxidoreductase (candidate dihydrolipoamide dehydrogenases) and an Rrf2 family repressor (distinct from cymR; NWMN_1528 [5]) that is related to SaiR, which was recently characterized in Bacillus subtilis (51). SaiR regulates the expression of Spx, an activator of the response to toxic oxidants; this is consistent with the orthogonal nature of the cellular response to RSS relative to ROS (see above).

Unregulated cst operon expression results in repression of staphylococcal toxin genes. Further consideration of the genes that are downregulated in the ΔcstR

| Locus tag (Newman) | Locus tag (N315) | Gene name | Fold induction (S2/H11002) | Function | Plus CP treatmentb (fold increase)? | ΔcstR regulation?
|-------------------|-----------------|-----------|--------------------------|----------|-------------------------------------|------------------|
| NWMN_2074 | SA0704 | 6.3 | 5.9 | Hypothetical | | |
| NWMN_2086 | SA1984 | 15.1 | | Alkaline shock protein 23 | | |
| NWMN_2087 | SA1985 | 18.3 | | Hypothetical (79 residues; COG5547) | | |
| NWMN_2088 | SA1986 | 11.8 | | Hypothetical | | |
| NWMN_2091 | SA1989 | 10.9 | | Hypothetical (quinone oxidoreductase) | | |
| NWMN_2109 | SA2006 | 12.2 | | Truncated MHC class II analog protein | | |
| NWMN_2180 | SA2075 | 13.3 | | Formate dehydrogenase accessory protein | | |
| NWMN_2209 | SA2101 | 10.7 | | Hypothetical | | |
| NWMN_2210 | SA2102 | 5.1 | | Formate dehydrogenase-like | | |
| NWMN_2229 | SA2119 | 15.2 | | Hypothetical | | |
| NWMN_2273 | SA2161 | 13.6 | | Acetyltransferase, GNAT family protein | | |
| NWMN_2274 | SA2162 | 13.6 | | Pyridine nucleotide-disulfide oxidoreductase (TrxB-like) | Yes (3.4) | |
| NWMN_2282 | SA2170 | 12.3 | | Hypothetical | | |
| NWMN_2306 | SA2184 | 32.0 | | Zinc-binding lipoprotein, AdcA-like | Yes (12.1) | |
| NWMN_2359 | SA2250 | 5.6 | | Major facilitator superfamily (MFS) | Yes (66.1) | |
| NWMN_2360 | SA2251 | 7.2 | | ABC transporter; cobalt-nickel | Yes (108) | |
| NWMN_2361 | SA2252 | 7.9 | | ABC transporter; cobalt-nickel | Yes (107) | |
| NWMN_2362 | SA2253 | 9.0 | | ABC transporter; cobalt-nickel | Yes (104) | |
| NWMN_2363 | SA2254 | 6.5 | 3.7 | ABC transporter; cobalt-nickel | Yes (101) | |
| NWMN_2364 | SA2255 | 6.6 | 5.0 | ABC transporter; cobalt-nickel | Yes (199) | |
| NWMN_2365 | SA2256 | 13.8 | 10.0 | Hypothetical (NWMN_2367–2365 operon) | Yes (504) | |
| NWMN_2366 | SA2257 | 12.1 | 15.7 | Hypothetical, epimerase-like | Yes (445) | |
| NWMN_2367 | SA2258 | 11.2 | 14.2 | Hypothetical | Yes (346) | |
| NWMN_2368 | SA2259 | 8.9 | | Hypothetical | | |
| NWMN_2369 | SA2260 | 15.1 | | Short-chain dehydrogenase | | |
| NWMN_2402 | SA2294 | 3.1 | 6.0 | Gluconate kinase | Yes | |
| NWMN_2403 | SA2295 | 6.9 | 5.5 | Gluconate operon repressor | Yes | |
| NWMN_2414 | SA2304 | 2.1 | 12.2 | Fructose-1,6-bisphosphatase | | |
| NWMN_2434 | SA2323 | 5.0 | | Hypothetical | | |
| NWMN_2435 | SA2324 | 11.0 | | Hypothetical | | |
| NWMN_2436 | SA2325 | 10.5 | | Hypothetical | | |
| NWMN_2456 | SA2343 | 3.0 | | Hypothetical (63 residues) | | |
| NWMN_2457 | SA2344 | copA | 68.3 | Cu(I)-specific P-type ATPase efflux transporter | | |
| NWMN_2458 | SA2345 | copZ | 26.1 | Cu(I) chaperone | | |
| NWMN_2461 | SA2348 | ctrM | 5.6 | Squalene synthase | | |
| NWMN_2462 | SA2349 | ctrN | 9.0 | Squalene synthase | | |
| NWMN_2463 | SA2350 | 13.6 | | Glycosyl transferase, group 2 family protein | | |
| NWMN_2464 | SA2351 | ctt | 4.6 | Phytoene dehydrogenase | | |
| NWMN_2479 | SA2366 | 6.8 | | Amidoxydrolase family protein | | |
| NWMN_2480 | SA2367 | 11.5 | | α/β hydrolase family protein | | |
| NWMN_2481 | SA2368 | 2.4 | 1.7 | Putative COG0523 GTPase | Yes (43.2) | |
| NWMN_2482 | SA2369 | 4.0 | 2.6 | Hypothetical | Yes (83.1) | |
| NWMN_2483 | SA2370 | 5.5 | 5.0 | FAD-dependent pyridine nucleotide disulfide oxidoreductase | Yes (152) | |

*a*Fold induction is shown where S2/H11002 induction is ≥3.0-fold.

*b*See Table S1A.

*c*2-fold induction with NaHS in HNO induction is statistically significant (see Table S1A; adjusted P value, ≤0.05).

*d*Below indicated cutoff values but part of an operon harboring genes above the cutoff value.
| Locus tag (Newman) | Locus tag (N315) | Gene name | Fold repression (S²⁻) | Fold repression (Angeli's salt) | Function | CymR regulon? | ΔcstR regulation? |
|-------------------|------------------|-----------|----------------------|-------------------------------|----------|---------------|-------------------|
| NWMN_0115 SA0165 | NWMN_0116 SA0166 | 16.9      | 4.2                  | Hypothetical                  | +        |               |                   |
| NWMN_0117 SA0167 | 34.0             | 5.7       | ABC transporter: putative TauB (sulfonate) | + |               |                   |
| NWMN_0118 SA0168 | 11.5             | 5.1       | ABC transporter: putative TauA | + |               |                   |
| NWMN_0119 SA0169 | 13.0             | 6.6       | ABC transporter: putative TauC | + |               |                   |
| NWMN_0143 SA0198 | 11.9             | 3.1       | ABC transporter: peptide (ATPase) | + | –/– down |                   |
| NWMN_0144 SA0199 | 9.4              | 11.1      | ABC transporter: peptide (permease) | – | –/– down |                   |
| NWMN_0145 SA0200 | 11.8             | 9.4       | ABC transporter: peptide (permease) | – | –/– down |                   |
| NWMN_0146 SA0201 | tlp              | 6.0       | Putative RGD-containing lipoprotein | – |               |                   |
| NWMN_0374 SA0368 | tcyP             | 7.5       | 4.4                  | Putative cystine transporter homolog | + |               |                   |
| NWMN_0391 SA0385 | ssi11nm          | 3.3       | 5.4                  | Superantigen-like protein 4 | – | Down |                   |
| NWMN_0400        | ssi11nm          | 3.4       | 7.1                  | Hypothetical                  | – | –/– down |                   |
| NWMN_0423 SA0417 | 2.3              | 5.4       | Sodium-dependent symporter | – | –/– up |                   |
| NWMN_0424 SA0418 | cysM             | 5.7       | Cystathionine-β-synthase (CBS) | + |               |                   |
| NWMN_0425 SA0419 | metB             | 8.5       | Cystathionine-γ-lyase (CSE) | + |               |                   |
| NWMN_0426 SA0420 | 13.0             | 11.6      | ABC transporter: peptide (ATPase) | – | –/– down |                   |
| NWMN_0427 SA0421 | 13.9             | 11.9      | ABC transporter: peptide (permease) | – | –/– down |                   |
| NWMN_0428 SA0422 | 9.9              | 4.1       | ABC transporter: peptide (substrate binding) | – | –/– down |                   |
| NWMN_0475 SA0471 | cysK             | 10.4      | Cysteine synthase (OAS + H₂S → acetate + Cys) | + |               |                   |
| NWMN_0557 SA0551 | 7.5              | Up 4.6    | Putative pyridine nucleotide disulfide oxidoreductase | – |               |                   |
| NWMN_0558 SA0552 | 16.6             | RtfA family repressor (related to Z. subtilis SaliR) | – |               |                   |
| NWMN_0605 SA0641 | mgrA             | 5.1       | MarR family repressor MgrA | – |               |                   |
| NWMN_0757        | 2.3              | 28.8      | Secreted von Willebrand factor-binding protein | – | –/– down |                   |
| NWMN_0759        | 3.0              |           | Hypothetical | – | –/– down |                   |
| NWMN_0766 SA0751 | 4.2              |           | Hypothetical | – |               |                   |
| NWMN_0813 SA0804 | 5.8              |           | Sodium/proton antipporter family protein | – |               |                   |
| NWMN_0831 SA0821 | argH             | 3.0       | Argininosuccinate lyase (Arg biosynthesis) | – | –/– down |                   |
| NWMN_0832 SA0822 | argG             | 6.2       | Argininosuccinate synthase (Arg biosynthesis) | – | –/– down |                   |
| NWMN_0907 SA0989 | 6.5              |           | Hypothetical | – |               |                   |
| NWMN_1047 SA0983 | isdG             | 3.1       | Heme-degrading monooxygenase | – |               |                   |
| NWMN_1066 SA1000 | 5.1              |           | Hypothetical (fibrinogen binding) | – |               |                   |
| NWMN_1068 SA1002 | 5.6              |           | Hypothetical | – |               |                   |
| NWMN_1069 SA1003 | 3.4              |           | Hypothetical (fibrinogen binding) | – |               |                   |
| NWMN_1070 SA1004 | 6.9              |           | Hypothetical (fibrinogen binding) | – |               |                   |
| NWMN_1132 SA1275 | 5.0              |           | Hypothetical | – |               |                   |
| NWMN_1174 SA1674 | tcyC             | 1.8       | 7.1                  | ABC transporter: cystine (ATPase) | + | Down |                   |
| NWMN_1175 SA1675 | tcyB             | 1.6       | 6.7                  | ABC transporter: cystine (permease) | + | Down |                   |
| NWMN_1176 SA1676 | tcyA             | 1.2       | 4.8                  | ABC transporter: cystine (binding) | + |                   |
| NWMN_1187 SA1755 | chp              | 32.7      | Chemotaxis-inhibiting protein | – |               |                   |
| NWMN_1195 SA1849 | 9.8              | 4.4       | TusA-like (SirA/YedF/YeeD) protein | + |               |                   |
| NWMN_1195 SA1850 | 14.2             | 5.0       | Putative thiosulfate (TS) importer | – |               |                   |
| NWMN_2049 SA1949 | cztA             | 15.9      | Zinc-specific repressor (Anr family) | – |               |                   |
| NWMN_2050 SA1950 | cztB             | 14.0      | Zinc cation diffusion facilitator (CDF) transporter | – |               |                   |
| NWMN_2075 SA1976 | 11.8             |           | Hypothetical | – |               |                   |
| NWMN_2186 SA2080 | ydB              | 9.3       | Putative CiaA; acyl (butyryl)-CoA dehydrogenase | + |               |                   |
| NWMN_2199 SA2093 | 2.1              | 4.2       | Secretory antigen precursor SsaA | – |               |                   |
| NWMN_2200 SA2094 | nhcA             | 8.4       | Sodium/proton antipporter family, NhaC | – |               |                   |
| NWMN_2201 SA2095 | 2.1              | 9.5       | Dehydrogenase family protein | – |               |                   |
| NWMN_2203        | 6.5              |           | Secretory antigen precursor SsaA | – |               |                   |
| NWMN_2265 SA2153 | 5.5              |           | Hypothetical | – |               |                   |
| NWMN_2311 SA2200 | 6.1              |           | ABC transporter: amino acid (ATPase) | – |               |                   |
| NWMN_2312 SA2201 | 5.3              |           | ABC transporter: amino acid (permease) | – |               |                   |
| NWMN_2313 SA2202 | 10.1             |           | ABC transporter: amino acid (substrate binding) | – |               |                   |
| NWMN_2317 SA2206 | sbi              | 5.0       | Immunoglobulin G-binding protein | – |               |                   |
| NWMN_2470 SA2357 | 5.2              |           | Hypothetical (regulatory protein) | – |               |                   |
| NWMN_2472 SA2359 | 7.0              |           | Hypothetical (just upstream of dtr) | – | –/– down |                   |
| NWMN_2473 SA2360 | 5.4              |           | Hypothetical (just upstream of dtr) | – | –/– down |                   |
| NWMN_2577 SA2471 | hisG             | 7.9       | ATP phosphoribosyltransferase C subunit (His biosynthesis) | – |               |                   |
| NWMN_2578 SA2472 | hisZ             | 6.0       | ATP phosphoribosyltransferase R subunit (His biosynthesis) | – |               |                   |

*Fold induction shown if S²⁻ induction is ≥3-fold.
*CymR regulon as determined previously on TSB plus 2.0 mM cysteine (3).
*Proposed to be involved in glutathione (GSH) assimilation and degradation (3).
*Approximately 2-fold repressed (downregulated [down]) or activated (upregulated [up]) in the ΔcstR strain (see Tables 3 and 4 for a list of genes differentially expressed in the ΔcstR strain), with adjusted P value of <0.05.
*At least 2-fold induction with NaHS (see Table S1A; adjusted P value of <0.05) in HNO induction is significant.
*The entire ida operon is modestly (≥2-fold) repressed (see Table S1A for a complete listing of these genes).
strain (Table 4) revealed uniform repression of the expression of staphylococcal exotoxins, encoded by genes NWMN_0388 to NWMN_0397 (ss1nm to ss10nm). There is also repression of additional toxin genes, including NWMN_1075 to NWMN_1077 and the gene encoding exfoliative toxin A (eta), a major histocompatibility complex (MHC) class II analog that impacts T-cell function and proliferation, and a gene encoding a formyl peptide receptor-like 1 inhibitory factor which inhibits the activation of neutrophils and monocytes. In addition, genes responsible for extracellular adhesion, including those encoding clumping factor A (clfA) and an extracellular matrix protein (ssp), and genes required for anaerobic growth and antibiotic resistance are also repressed. Some of these genes are direct targets of MgrA, which harbors a single regulatory cysteine residue characterized by a range of oxidative modifications in cells (52, 53).

Other genes that are downregulated in the ΔcstR strain are upregulated during anaerobic growth of wild-type cells (COL) (54), while a significant fraction of these genes are similarly repressed upon treatment of S. aureus Newman cells with antimicrobial peptides (55). These genes include those encoding the formate acetyltransferase (pflB) and the formate acetyltransferase-activating enzyme; a formate-nitrate transporter (NWMN_0247) and the nitrite reductase transcriptional regulator (nirR); and the anaerobic ribonucleotide reductase (nrdGD). In addition, genes encoding a candidate flavohemoglobin (NWMN_0175), NO-inducible l-lactate dehydrogenase (ldh1), and l-lactate permease 2, as well as alcohol dehydrogenase (adh1), and genes associated with amino acid metabolism are also significantly repressed in the ΔcstR strain.

Comparison of the ΔcstR strain and the wild-type strain results seen at times shortly following an acute-phase sulfide shock shows that their transcriptomic responses diverged considerably beyond the approximately 10 genes that were similarly upregulated or downregulated under both conditions (Fig. 2A and Tables 1 to 4). Sulfide-stressed cells differ strongly from the ΔcstR strain in the relative concentrations of low-molecular-weight thiol persulfides (organic RSS) (26), which are strongly elevated relative to the ΔcstR strain but reduced relative to the untreated wild-type strain (see below). This suggests the possibility that ambient RSS might directly impact gene expression through oxidative modification of one or more cysteine-containing global regulators, with genes required for infection, dissemination, adhesion, antibiotic resistance, and anaerobic growth largely repressed in the ΔcstR strain relative to the wild-type strain.

Transcriptomic profiling of the effects of an exogenous HNO donor, Angeli’s salt. Emerging evidence suggests that many of the properties attributed to H₂Sa signaling molecule may derive from a significant increase in the levels of organic and inorganic polysulfide species and, in some cases, of HNO (15). HNO reacts rapidly with
LMW (and protein) thiols (10, 19). This leads to disulfide bond formation in the presence of resolving thiol with the release of hydroxylamine (10) and sulfanamides [RS(O)NH₂], which are in turn slowly reduced by cellular thiols (56). Indeed, baker's yeast encodes an enzyme that catalyzes the NADPH-dependent reduction of the S-nitrosoglutathionederived glutathione sulfanamide to reduced glutathione, which accumulates in cells under conditions of NO· stress (57). We therefore tested the effects of HNO added to aerobically growing cells by the use of Angeli's salt (AS), i.e., dinitrogen trioxide dianion (Na₂N₂O₃), which undergoes cleavage to yield HNO and nitrite (NO₂⁻) (Fig. 1C). In
TABLE 3 List of genes significantly (≥3.5-fold) upregulated in the ΔcstR strain relative to the wild-type strain

| Locus tag (Newman) | Locus tag (N315) | Gene name | Fold induction | Function | Sulfide stress?a |
|--------------------|-----------------|-----------|----------------|----------|-----------------|
| NWMN_0025 | SA0079 | tauE | 15.7 | Hypothetical | – |
| NWMN_0026 | SA0080 | cstA | 43.0 | Putative sulfonate/Ts effluxer | – / +b |
| NWMN_0028 | SA0082 | cstB | 70.2 | Multidomain sulfurtransferasea | + |
| NWMN_0029 | SA0084 | sfr | 46.5 | Persulfide dioxygenase-sulfur transferaseb | + |
| NWMN_0031 | SA0087 | gntK | 5.8 | Hypothetical | – |
| NWMN_0113 | SA0162 | alda | 5.3 | Aldehyde dehydrogenase-like | + |
| NWMN_0151 | SA0206 | putA | 7.7 | Putative ABC sugar transporter (NWMN_0151–0154) | – |
| NWMN_0329 | SA0325 | gntR | 5.2 | Glycerol-3-phosphate transporter | – |
| NWMN_1207 | SA1140 | glpF | 16.7 | Glycerol uptake facilitator protein | + |
| NWMN_1378 | SA1301 | ndk | 3.9 | Nucleotide diphosphate kinase | – |
| NWMN_1415 | SA1339 | marR | 4.2 | Malate operon repressor | + |
| NWMN_1658 | SA1585 | putA | 5.6 | Proline dehydrogenase | – |
| NWMN_1674 | SA1601 | crcB | 4.1 | Camphor resistance protein | – |
| NWMN_1681 | SA1609 | pckA | 3.5 | Phosphoenolpyruvate carboxylase | – |
| NWMN_2074 | SA2207 | hiaA | 7.4 | Hypothetical (conserved; 86 residues) | – |
| NWMN_2318 | SA2294 | gntK | 6.1 | Guanulate kinase | + |
| NWMN_2403 | SA2295 | gntR | 5.4 | Guanulate operon repressor | + |
| NWMN_2510 | SA2406 | gntA | 12.0 | Hypothetical; putative transporter protein (gluaronic acid) | – |
| NWMN_2513 | SA2408 | gntB | 6.2 | Glycine betaine aldehyde dehydrogenase GbsA | – |

aSee Table 1.

bAt least 2-fold induction with NaHS (see Table S1A; adjusted P value of ≤0.05).

contrast to the minimal induction (Fig. 5S) or absence of induction of the cstR-regulated genes resulting from the addition of an NO• donor or nitrite, respectively (29), we found that AS significantly induced cstA expression in a quantitative reverse transcription-PCR (qRT-PCR) experiment (Fig. 5A). Further, a ΔcstR strain complemented with a mutant CstR unable to sense persulfides cannot be induced under the same conditions, suggesting that HNO impacts CstR function. These cells also exhibit a dramatic growth defect relative to the wild-type strain stressed with HNO (Fig. 5B). We next tested if HNO is capable of reacting directly with CstR thiols, leading to disulfide bond formation (10), which would induce derepression of CstR-regulated genes (29). Treatment of reduced CstR with Angeli’s salt in vitro does indeed yield CstR characterized by an interprotomer disulfide bond between C31 and C60 confirmed by both liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) and LC-tandem MS (LC-MS/MS), with no cross-linked products obtained upon incubation with sodium nitrite (Fig. 5C). This reveals that HNO is capable of impacting CstR-regulated transcription directly by forming a cross-linked CstR which has lower affinity for operator DNA (28).

A transcriptomic analysis of all genes induced (Table 1) or repressed (Table 2; see Table S1A for a complete list) by AS reveals a significant genome-wide change in cellular transcription (Fig. 6). We focused our attention on those genes whose expression changed by approximately 1 standard deviation from the mean induction level (approximately 10-fold; Table 1) or the mean repression level (approximately 5-fold; Table 2). Beyond the cst operon, there was a subset of genes that were upregulated by both HNO and sulfide treatment relative to the results seen with untreated wild-type cells (Table 1 and Fig. 7). These genes included a gene that is part of the CymR regulon, alda (NMWN_0113), which is among the most highly (56-fold) upregulated genes in the genome; cntKLMB, encoding a broad-spectrum metallophore biosynthetic cluster (48) and among the genes most highly upregulated by sulfide treatment (Table 1); and NWMN_0815, a gene encoding a putative pyridine nucleotide (FAD) disulfide reductase (Fig. 7). These genes are also associated with the zinc limitation, CP-mediated transcriptomic response (Table 1) (Fig. 2), which suggests that zinc may well become limiting as a result of intracellular chelation of the metal upon AS treatment (58). Several other genes involved in the metal limitation response, e.g., rpmG2, rpsN2, cntAB,
and NWMN_2483, are also detectably induced by HNO stress but to a level that is lower than that due to sulfide stress. Consistent with this, AS treatment leads to a detectable (approximately 3-fold) decrease in the Zn concentration and to a smaller (1.8-fold) decrease in the Cu concentration in the growth medium, suggesting that HNO, like sulfide itself, may be capable of reducing the bioavailability of transition metals in cells.

The remaining genes in the far larger panel that are upregulated by AS treatment but unaltered by sulfide treatment show some overlap with respect to those genes induced by nitrite in nitrate-respiring, nitrite-stressed cells (59). Most notable is the massive upregulation of the copAZ operon, encoding a Cu(II)-transporting P-type ATPase effluxer and a Cu(II) chaperone, both under the transcriptional control of Cu(I)-sensing repressor CsoR (NWMN_1992) (28). CsoR is a paralog of CstR and is unresponsive to sulfide stress in cells (28). This suggests that HNO-mediated modifica-
tion of LMW thiols, which maintain bioavailable Cu(II) at low levels, may result in the displacement of Cu(II) from these stores, which is then sensed by CsoR. Indeed, analysis of total cell-associated metal levels by inductively coupled plasma-mass spectrometry (ICP-MS) reveals a significant and specific increase in levels of cellular Cu (Fig. S7), consistent with this hypothesis. The remaining AS-affected genes represent an Fe overload response, the origin of which is probed below, coupled with a PerR-regulated ROS response and induction of the stress-associated CtsR regulon (clpB, clpC) and a DNA damage response (uvrBC) (Fig. S2). These transcriptomic changes, taken collectively, are consistent with an acute-phase combined ROS/RNS stress response induced by AS treatment (Fig. S2).

AS also represses a number of virulence factors, including superantigen-like proteins; secreted von Willebrand factor-binding protein; proteins encoded by NWMN_1066 and NWMN_1070, which are predicted to be fibrinogen binding, chemotaxis-inhibiting proteins; secretory antigen precursor SsaA; and immunoglobulin G-binding protein. We globally compared those genes whose expression is affected by AS to genes encoding other virulence factor regulons, including those encoded by sarA (60), sarZ (61), mgrA (62), sigB (63), and rot (64). We generally found no clear correlation of upregulation or downregulation between AS-responsive genes and those encoding these virulence regulons, with the exception of sigB, where many of the transcriptomic changes that were common under these two conditions occurred in the same direction (Fig. S8). SigB is an alternative sigma factor (σH9268) that controls the response to heat stress, oxidative stress, and antibiotic stress and appears to be linked to intracellular survival during chronic infection (65) while impacting the expression of genes encoding other virulence regulators, including sarA, sarS, and arlRS, a two-component system of autolysis (63). We note that most of the virulence factors repressed by AS treatment are not regulated by sulfide stress, suggesting that host NO-derived RNS, e.g., HNO (Fig. 1B), likely have important regulatory roles in virulence expression distinct from that of sulfide.

**RSS profiling in sulfide-stressed versus Angeli’s salt-stressed cells.** We previously employed a fluorescence-based analytical method to detect and quantify LMW monobromobimane (mBBr)-derivatized sulfur-containing metabolites in cells (29). Here, we extended this method to incorporate ratiometric (32S/34S) tandem mass spectrometry, in which the concentrations of all organic thiols and of per- and polysulfides relative to those seen with an internal standard can be detected in a single experiment. We quantified bacillithiol persulfide (BSSH), cysteine persulfide (CysSSH), and coenzyme A persulfide (CoASSH) in the wild-type and ΔcstR S. aureus strains and in sulfide- or AS-stressed wild-type S. aureus. The ΔcstR strain showed slightly lower levels of LMW

![RNAseq transcriptomic analysis of Staphylococcus aureus strain Newman cells treated with sulfide (red filled circles) versus Angeli’s salt (green filled circles). The fold change in expression for each locus tag is indicated (Tables 1 to 2; Table S1A). Gene names are indicated where known. Expression of those highlighted in red text was significantly induced or repressed under both experimental conditions; expression of those highlighted in light blue was induced during calprotectin (CP) and Angeli’s salt (nitroxyl) treatment.](msphere.asm.org)
persulfides (Fig. 5C), consistent with an RSS clearance function of the cst operon-encoded enzymes. As predicted from the transcriptomics experiments, both sulfide stress (Fig. 5C) and AS treatment (Fig. 5D) caused an increase in cellular levels of LMW thiol persulfides, while nitrite, one of the AS decomposition products (Fig. 1B and C), did not (Fig. 5E). These results directly implicate HNO or a downstream reaction product(s) in this cellular increase in RSS (Fig. 1B). Thus, HNO may directly react with CstR thiol s to induce transcriptional derepression of the cst operon (Fig. S6) or, alternatively, may induce higher levels of cellular LMW persulfides which are in turn sensed by CstR.

To further explore this, we reasoned that HNO could directly mediate an increase in cellular concentrations of RSS via at least two possible mechanisms. One is a mechanism by which HNO upregulates the expression of a sulfide biogenesis pathway(s), e.g.,
one involving CBS (CysM) and CSE (MetB). An alternative possibility is that AS-derived HNO disassembles Fe-S clusters directly or reacts with molecular oxygen to create ONOO⁻ (21, 66), which is known to be capable of destabilizing Fe-S clusters in proteins (Fig. 1C) (24). Both would lead to an increase in endogenous sulfide as well as chelatable Fe(II) levels in the cell. Human CBS binds heme via coordination by Cys52 and His65 in an N-terminal heme-binding domain, and RNS are capable of regulating CBS activity by changing the oxidation state of the heme (67). However, this domain is not conserved in S. aureus CysM (alignment not shown). Consistent with this, RSS levels are in fact transiently elevated in a ΔcysM ΔmetB strain by AS treatment, albeit to an extent lower than that seen in the wild-type strain (Fig. 5F).

To measure the effect of molecular oxygen on this AS (HNO) stress-induced increase in RSS, we carried out this experiment under microaerophilic conditions, where oxygen levels are significantly decreased by growing static cultures in tightly capped tubes, with 5 mM nitrate as the electron acceptor of anaerobic respiration. AS-inducible increases in RSS were observed in these cultures (Fig. 5G) that were nearly equal to those seen with aerobically grown cells (Fig. 5D). Cells grown aerobically and treated with a burst of ONOO⁻ also induced organic RSS but to a level that was lower overall than that seen with AS treatment (Fig. 5H). This result may have been related to the vanishingly short lifetime of ONOO⁻ under these conditions. We propose that the presence of HNO or a downstream product(s) (Fig. 1B) leads to the disassembly of Fe-S clusters and to a corresponding increase in endogenous sulfide and RSS levels, with the oxidant ONOO⁻ also capable of this chemistry (21, 66). In support of this idea, although the total cell-associated Fe level did not change (Fig. S7), our transcriptomic analysis strongly suggests an increase in the level of cytoplasmic chelatable iron, as exemplified by the upregulation of genes encoding the Fe-S cluster biogenesis pathway (sufC, sufD, sufS, NIFU, and sufB), the iron-storage proteins ferritin (ftn) and Dps (dps), and the iron uptake repressor (fur; NWMN_1406), which could chelate cellular Fe and further repress Fe import. Regardless of the precise mechanism involved, these data, taken collectively, suggest that HNO, as a principal product of H₂S/NO· interplay (15, 68), directly impacts endogenous sulfur speciation and global gene expression in S. aureus.

Conclusions. In this report, we show that the cellular transcriptomic response of the major human pathogen S. aureus to the effects of exogenous sulfide exhibits some parallels to the cellular response to Angeli’s salt, a commonly used HNO donor. Under these conditions, HNO appears to signal partly through perturbations in sulfur speciation in cells, as anticipated by much of the small-molecule chemistry that has been reported for this primary product of RNS/RSS interplay (15, 68). The origin of both effects derives in part from a significant increase in levels of cellular RSS (Fig. 5). The RSS-regulated transcriptomic response is opposite that induced by oxidants and suggests the possibility that bacterial cells can manage intracellular RSS as a means to provide protection against irreversible oxidation by oxidative stressors, as has been previously established in mammalian cells (25, 69). In contrast, decreases in levels of ambient RSS induced by overexpression of the cst-encoded sulfide oxidation system in a ΔcstR strain repressed virulence gene expression, i.e., expression of genes required for infection, dissemination, and adhesion to cells.

These results support the use of cellular RSS as a readily deployable chemical strategy to impact the physiological state of a bacterial community (32) and otherwise to mediate an adaptive response to changes in host microenvironments mediated by ROS and RNS. Indeed, multiple reactive small-molecule stressors often have a synergistic effect on microbial killing (70) and it is unusual for a pathogen to encounter a single stressor at a site of infection. This deployment of RSS, however, must be tightly managed, as intracellular sulfide and HNO (or downstream reaction products) significantly reduce zinc bioavailability, while HNO potentially increases levels of cytoplasmic free Cu (Fig. S7) and free Fe, the latter possibly via destruction or inhibition of assembly of Fe-S clusters. It is interesting in this context that one of the cst operon-encoded proteins, CstA, is capable of stripping the active site persulfide from the major S. aureus.
cysteine desulfurase SufS in a persulfide transfer reaction (30). This might provide a means to divert sulfur flow from Fe-S protein biogenesis to cellular RSS as a protective mechanism to minimize exposure to oxidative damage mediated by free Fe. This is consistent with the strong upregulation of Fe- and PerR-regulated ROS-inducible genes, including those encoding catalase (katA), flavohemoglobin (hmp), a candidate peroxidoredoxin (bcp), alkylhydroperoxidases (ahpD, ahpF), a candidate nitroreductase, and the iron-storage protein Dps (dps) (Table S1A; Table 1; Fig. 7). These studies have set the stage for further elucidation of H$_2$S/NO cross talk and proteome S-sulfhydration-based pathways in aerobically versus anaerobically growing bacterial cells.

**MATERIALS AND METHODS**

**Chemicals and reagents.** A5 (82230) and sodium peroxynitrite (81565) were purchased from Cayman Chemical. Monodromobimine (M-20381) was purchased from Invitrogen. Sulfur-34 metal (SLM-1085-PK) used for Na$_3$S$_4$ synthesis was purchased from Cambridge Isotope Laboratories, Inc. Sodium sulfide (407410), sodium nitrite (237213), sodium nitrate (55506), oxidized coenzyme A (C2643) used to synthesize the CoASSH standard, L-cysteine (C8755) used to synthesize the CysSSH standard, and other reagents were purchased from Sigma-Aldrich.

**S. aureus RNAseq and qRT-PCR experiments.** Sample collection, RNA extraction, and the procedure for qRT-PCR experiments were described previously (29). The primers used in qRT-PCR experiments are listed in Table S1B in the supplemental material. Sequencing reads were trimmed using Trimmomatic (version 0.33) with the following parameters: ILLUMINACLIP:adapter.fa:2:20:6 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:20 MINLEN:35. The trimmed reads were mapped onto the *Staphylococcus aureus* subsp. *aureus* strain Newman genome using bowtie2 (version 2.1 with default parameters) (72). Read counts for genes and intergenic intervals were calculated using a custom perl script. The resulting gene/interval counts were used to conduct differential expression analysis using the program DESeq2 algorithm (73) with default parameters. For transcriptome sequencing (RNAseq) analysis of calprotectin (CP)-treated cultures, bacteria were cultured overnight in Chelex-treated RPMI medium plus 1% Casamino Acids supplemented with 1 mM MgCl$_2$, 100 mM CaCl$_2$, and 1 mM FeSO$_4$. These samples were then back-diluted 1 to 100 into growth medium containing 38% tryptic soy broth (TSB); 72% calprotectin buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 3 mM CaCl$_2$, 10 mM β-mercaptoethanol), supplemented with 1 μM Zn and 1 μM Mn. The bacteria were grown to an exponential-phase optical density at 600 nm (OD$_{600}$) of 0.25 to 0.4 in the presence and absence of 960 μg/ml CP. Samples were harvested and processed as previously described (29), with the exception that RNA was isolated using the method reported by Collins et al. (74). The mean level of induction of all genes that showed an increase in expression of ≥2.0-fold at an adjusted P value of ≤0.05 in the CP-treated cells was 20-fold (±59-fold), with a median genome-wide level of induction of 4.4-fold (330 genes). The mean level of expression for all genes that were downregulated as a result of CP treatment was 7.4-fold (±8.0-fold), with a median level of genome-wide repression of 4.3-fold (289 genes). The results of all RNAseq experiments have been deposited in the GEO database (see below).

**CstR reaction with AS.** The purification of CstR was carried out as described previously (29). A 200-μl volume of 25 μM apo- and reduced CstR was incubated anaerobically with a 20-fold molar excess over levels of thiol of sodium nitrite or Angeli’s salt in 25 mM Tris (pH 8.0)–500 mM NaCl–5 mM EDTA at room temperature. After 30 min, 100 μl of the reaction mixture was transferred to a vial, the vial was tightly capped, and the contents of the vial were injected in LC-ESI-MS experiments to determine if cross-linked CstR was formed, as previously described (29). The remainder of each sample was alkylated with iodoacetamide, digested by trypsin, and sequenced to further confirm the formation of a disulfide bond.

**Bacterial growth curves and cell culture.** The wild-type *S. aureus* Newman strain and the ΔcstR CstRΔ31A/C60A strain were described previously (29). The ΔcysM ΔmetB strain was constructed by allelic exchange (75). The fragment upstream of cysM was amplified using primers 5′-TGAGCTCCGAACCCGGAACAGTTGCGGCACTATGAGC-3′ and 5′-TCTAGGCTAGCTAGCAATTAACTCATAAATGATATACGATGC-3′; a spectinomycin resistance cassette was amplified from pSPC using primers 5′-GCTAGACATGGCTACG-3′ and 5′-GCTAGACATGGCTACG-3′; and the fragment downstream of metB was amplified using primers 5′-TCCTCAATTAGCTAGCAGCCAAGCAGCATCAATCTTATTTTC-3′ and 5′-TCCTCAATTAGCTAGCAGCCAAGCAGCATCAATCTTATTTTC-3′; and the fragment downstream of metB was amplified using primers 5′-TCCTCAATTAGCTAGCAGCCAAGCAGCATCAATCTTATTTTC-3′ and 5′-TCCTCAATTAGCTAGCAGCCAAGCAGCATCAATCTTATTTTC-3′. These fragments were assembled with pKR1 linearized by digestion with EcoRI and KpnI using NEB Gibson assembly and were integrated into the *S. aureus* genome. The lesion was then transduced into a clean *S. aureus* Newman background using bacteriophage φ85, and transductants were selected with spectinomycin at 1,000 mg/liter. All bacterial strains were grown overnight in TSB with 10 μg/ml chloramphenicol. Cells were pelleted and resuspended in Hussain-Hastings-White modified (HHWm) minimal media (76) supplemented with 50 μg/ml chloramphenicol and 0.5 mM thiousole as the sole sulfur source. For growth curve analyses, cultures were initiated at an OD$_{600}$ of 0.007 with or without 0.2 mM AS stress added to the growth medium. All aerobically grown cultures were grown at 37°C with shaking (200 rpm), with the OD$_{600}$ measured every hour from h2 to h10.

**Quantification of cellular LMW thiol persulfides.** Overnight *S. aureus* cells grown in TSB were diluted to an OD$_{600}$ of 0.02 in HHWm minimal medium (76) supplemented with 0.5 mM thiousole as the sole sulfur source and grown aerobically. When these cultures reached an OD$_{600}$ of 0.2, 0.2 mM disodium sulfide, AS, sodium nitrite, or sodium peroxynitrite was added. For microaerophilic conditions, 5 mM sodium nitrite was added as an electron acceptor to 50-ml tubes that were capped tightly without

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The tubes were opened for addition of AS and were gently inverted for mixing. Samples (5 ml plus 1 ml for protein quantification) were collected before (t = 0 min) and after addition of the stressor at the indicated times. Following addition of stressors and were centrifuged at 3,000 rpm for 10 min. The resulting pellets were washed with ice-cold phosphate-buffered saline (PBS), pelleted again by centrifugation (16,100 rpm for 5 min), and stored frozen at −80°C until use. Thawed cell pellets were resuspended in 100 µl monobromobimane (mBBr) labeling solution containing 20 mM Tris-HBr (pH 8.0), 50% acetonitrile, and 1 mM mBBr and subjected to three freeze-thaw cycles in liquid nitrogen in the dark in screw-cap tubes (77). Cell debris was removed by centrifugation, and the supernatant was transferred to a tube containing 100 µl 15 mM methanesulfonic acid (MA) to quench the labeling reaction (31). Finally, particulates were removed via passage through a 0.2-µm-pore-size centrifugal filter unit prior to injection into a liquid chromatograph mass spectrometry (LC-MS) system for quantitation of LMW thiol and persulfides as follows.

Samples (10 µl) were injected into a Triart C18 column (YMC, Inc.) (50 by 2.0 mm inner diameter) and subjected to chromatography on a Waters Acquity Ultra Performance Liquid Chromatography (UPLC) I-class system, using a methanol-based gradient system (for solvent A, 10% methanol and 0.25% acetic acid, pH 3.0; for solvent B, 90% methanol and 0.25% acetic acid, pH 3.0) with the elution protocol at 25°C and a flow rate of 0.2 ml/min as follows: at 0 to 3 min, 0% B isocratic; at 3 to 7 min, 0% to 25% B, linear gradient; at 7 to 9 min, 25% B isocratic; at 9 to 12 min, 25% to 75% B, linear gradient; at 12 to 14 min, 75% to 100% B, linear gradient; at 14 to 14.5 min, 100% B isocratic, followed by reequilibration to 0% B. Quantitation of LMW thiols and persulfides was carried out with a Waters Synapt G2S mass spectrometer by spiking in a specific amount of authentic 34S-containing LMW persulfide standards synthesized with Na34S in place of Na32S (31) to achieve a typical 34S/32S persulfide ratio of approximately 0.2 to 1.0. To quantify the relative change in the BSH/BSSB ratio, the peak area of mBBr-derivatized BSH and the peak area of underivatized BSSB in each sample were obtained and normalized to that of a known concentration (peak area) of mBBr-labeled N-acetylcysteine (NAC), with the same concentration added to each sample. The change of the ratio was obtained by dividing the ratio seen under stress conditions by the ratio for the unstressed sample, with standard propagation of errors. Analysis of peak areas was performed in Masslynx (v 4.1) software, and the data were normalized to protein concentrations measured using a Bradford assay with bovine serum albumin (BSA) as the standard, as previously described (26). Data shown represent means and standard deviations of results from three biological replicates.

**Synthesis of isotope-labeled internal standards.** Na234S was synthesized using a published protocol (78). Bacillithiol persulfide (BS34SH), cysteine persulfide (Cys34SH), and coenzyme A persulfide (CoA34SH) were synthesized by reacting the appropriate oxidized disulfide (RSSR) with Na2S to obtain an equimolar mixture of the thiol RS– and the persulfide RSS– (16). Bacillithiol disulfide (BSSB; 5 mM), generously provided by M. Kiethy (Vanderbilt University), CoA disulfide (5 mM), or γ-cysteine (2.5 mM) was reacted anaerobically with a 5-fold molar excess of Na34S–300 mM degassed phosphate buffer (pH 7.4) at 30°C for 30 min. The concentration of persulfide in the final mixture was quantified by a cold cyanolysis assay (30), and the persulfide was diluted to 0.1 mM in 20 mM Tris-HBr (pH 8.0)–50% acetonitrile–2 mM mBBr and labeled as described above. Standard samples were used without further purification.

**Transition metal measurements.** Aliquots of growth medium with or without the addition of 0.2 mM sulfide or 0.2 mM AS were taken, centrifuged to remove any precipitates, diluted 10-fold in 2.5% HNO3, and analyzed using a PerkinElmer Elan II DRC ICP-MS system essentially as described in our previous work (79). To analyze the total cell-associated metal content, 5-ml cultures (OD600 of 0.2) were pelleted, the pellet was resuspended in 400 µl 30% nitric acid, and metal concentrations were determined as described above for the growth medium, with normalization to the amount of protein in each sample (in nanomoles per milligram of protein) as previously described (80). Metal concentrations were determined from a standard curve of 1 to 30 ppb metal stock solutions.

**Accession number(s).** The results of all RNAseq experiments have been deposited in the GEO database under GenBank accession number GSE99432.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at [https://doi.org/10.1128/mSphere.00082-17](https://doi.org/10.1128/mSphere.00082-17).
Sulfide-Nitroxyl Cross Talk in Staphylococcus aureus

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We declare that we have no competing financial interests.

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