HHS Public Access

Author manuscript

ACS Infect Dis. Author manuscript; available in PMC 2018 March 22.

Published in final edited form as:

ACS Infect Dis. 2017 October 13; 3(10): 744–755. doi:10.1021/acsinfecdis.7b00090.

Hydrogen Sulfide and Reactive Sulfur Species Impact Proteome *S*-Sulfhydration and Global Virulence Regulation in *Staphylococcus aureus*

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Abstract

Hydrogen sulfide (H_2S) is thought to protect bacteria from oxidative stress, but a comprehensive understanding of its function in bacteria is largely unexplored. In this study, we show that the human pathogen *Staphylococcus aureus* (*S. aureus*) harbors significant effector molecules of H_2S

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Author Contributions

D.P.G., J.C.T., and E.P.S. conceptualized and designed this collaborative study. H.P. and D.P.G. wrote the manuscript. H.P. performed and analyzed all RSS profiling experiments, performed the proteomics experiments, and carried out all biochemical assays. H.P., Y.Z., and J.C.T. analyzed the proteomics experiments. L.D.P. constructed the Δ*cysM*/ Δ*metB* and *trxP*::Tn(kan) *trxQ*::Tn(erm) mutant *S. aureus* strains and carried out all cell cytotoxicity assays using secretome fractions prepared by H.P. and analyzed by H.P. and Y.Z., under the supervision of E.P.S. T.E.K.-F. performed the Δ*cstR* infection under the supervision of E.P.S.

Notes

The authors declare no competing financial interest.

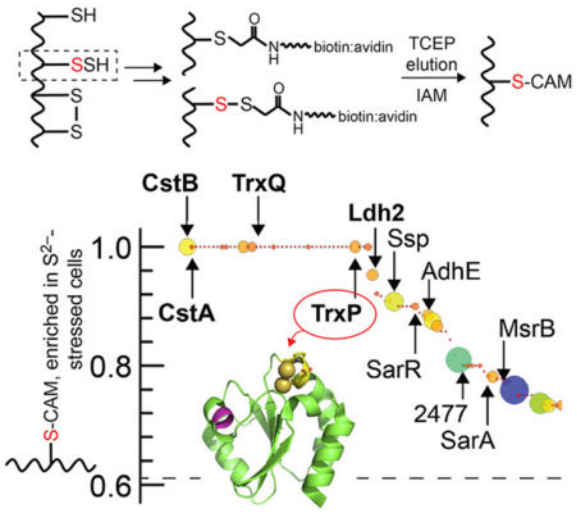
Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfec-dis.7b00090. Experimental procedures; concentrations of thiol and RSS in various *S. aureus* Newman strains; kinetic parameters of thioredoxin-like proteins; NWMN_0774 (TrxP) reduction of *S*-sulfhydrated Ldh2; cellular inorganic/organic RSS levels in *S. aureus*; summary of the *S*-sulfhydrated peptide analysis; LC-MS/MS analysis of selected *S*-sulfhydrated proteins; effect of RSS on MgrA function; secretome analysis of *S. aureus* FPR3757 strain; cytotoxicity assays of secretome fractions on human cells; quantitation of secretome proteins by mass spectrometry; examples of *S*-sulfhydrated nonconserved cysteines in protein structures (PDF)

Composite list of all proteins that are reproducibly identified as *S*-sulfhydrated in sulfide-stressed cells (XLSX)

signaling, reactive sulfur species (RSS), as low molecular weight persulfides of bacillithiol, coenzyme A, and cysteine, and significant inorganic polysulfide species. We find that proteome *S*-sulfhydration, a post-translational modification (PTM) in H_2S signaling, is widespread in *S. aureus*. RSS levels modulate the expression of secreted virulence factors and the cytotoxicity of the secretome, consistent with an *S*-sulfhydration-dependent inhibition of DNA binding by MgrA, a global virulence regulator. Two previously uncharacterized thioredoxin-like proteins, denoted TrxP and TrxQ, are *S*-sulfhydrated in sulfide-stressed cells and are capable of reducing protein hydrodisulfides, suggesting that this PTM is potentially regulatory in *S. aureus*. In conclusion, our results reveal that *S. aureus* harbors a pool of proteome- and metabolite-derived RSS capable of impacting protein activities and gene regulation and that H_2S signaling can be sensed by global regulators to affect the expression of virulence factors.

Graphical Abstract



Keywords

hydrogen sulfide; reactive sulfur species; thiol redox proteome; post-translational modification; secretome; S-sulfhydration reduction

Staphylococcus aureus (*S. aureus*) is a human commensal organism and nosocomial pathogen that is the causative agent of a range of illnesses, from minor skin infection to life-threatening diseases. The emergence of antibiotic-resistant strains of *S. aureus* such as methicillin-resistant *S. aureus* (MRSA) remains a significant threat to clinical medicine. The success of *S. aureus* is linked to its ability to express a vast array of virulence genes to allow adaption to host immune systems and survival in challenging microenvironments.[1] Although mammals have developed both innate and adaptive immune strategies to fight invasive microbes, *S. aureus* has coevolved with these host defense systems and secretes a variety of immune evasion determinants including staphylococcal super-antigen-like proteins to manipulate host immune responses.[2] The expression of a large number of virulence factors is coordinately regulated during pathogenesis and requires both two-component regulatory systems and members of the SarA protein family, comprising an interdependent and tightly

controlled network.[3] In addition to immunocytes, *S. aureus* encounters neutrophil-derived reactive oxygen species (ROS) including superoxide anion, hydrogen peroxide (H_2O_2), and hydroxyl radical (HO·).[4] Emerging evidence suggests that endogenous hydrogen sulfide (H_2S) protects cells against these oxidative stressors.[5–8]

Despite its historical recognition as a toxic gas and inhibitor of aerobic respiration,[9,10] endogenously synthesized H_2S (which we refer collectively to H_2S, HS^-, and S^{2-}) has recently been described as a signaling molecule or gasotransmitter with specific cellular and molecular targets in mammals.[11] It is generally accepted that H_2S fulfills its signaling function by inducing post-translational modification (PTM) of protein cysteine residues to create an *S*-sulfhydryl or persulfide moiety, RSSH, which can alter protein activity and influence diverse biological processes.[12] For example, *S*-sulfhydration of a conserved cysteine residue of pyruvate carboxylase increases its enzyme activity and contributes to gluconeogenesis.[13] *S*-Sulfhydration of cysteine residues of multiple Ca^{2+} channels maintains the Ca^{2+} flux in bone homeostasis,[14] while the activity of parkin, the ubiquitin E3 ligase associated with Parkinson's disease, is enhanced by *S*-sulfhydration.[15] However, H_2S itself cannot react with cysteine thiolates and is not a signaling species;[16] instead, H_2S leads to the production of more oxidized forms of sulfur, collectively termed reactive sulfur species (RSS) and including inorganic (S_n^{2-}, $n \geq 2$) and organic persulfides (RSS^-) and hydropolysulfides (RSS_n^-, $n > 1$), as true effector molecules of H_2S signaling in mammalian cells.[5,17] RSS generally contain one or multiple sulfane or "sulfur-bound" sulfur atoms[18] that are capable of oxidizing cysteine residues. It is hypothesized that RSS are generated either by direct attack of H_2S on low-molecule-weight (LMW) thiol disulfides or enzymatically via sulfide/quinone oxidoreductases (SQR).[19,20] RSS are maintained at micromolar levels in mammalian cells and animal tissues to allow H_2S signaling.[5]

In contrast to higher eukaryotes, direct support for sulfide-and RSS-based signaling in microbes is lacking beyond the per-and polysulfide-sensing transcriptional regulators that regulate the expression of genes generally encoding enzymes involved in sulfide oxidation.[21–23] These regulators sense RSS directly, which can be detected and quantified in bacterial cells.[20,24] However, little is known about what role H_2S and downstream RSS play in cellular sulfur speciation and if H_2S signaling impacts *S. aureus* pathogenesis and other biological processes. Sulfur metabolism is generally important for bacterial pathophysiology, and targeting microbial sulfur assimilation is a validated approach for the development of new antimicrobial agents.[25] Changes in sulfur metabolism influence the ability of *S. aureus* to form biofilms.[26] Cysteine availability also affects toxin synthesis of *Clostridium difficile*,[27] and endogenous production of H_2S is reported to protect *S. aureus* and other bacteria against oxidative and antibiotic stress.[7,8] Endogenous biogenesis of H_2S occurs in *S. aureus*, and the concentrations of sulfide and downstream inorganic and organic RSS can be controlled via genetic or chemical perturbation of endogenous or exogenous sulfide levels.[24] H_2S is produced primarily by the enzymes of the transsulfuration pathway,[28] cystathionine-β-synthase (CBS, encoded by *cysM*) and cystathionine-γ-lyase (CSE, *metB*). A dithiol-containing repressor of the *cst* operon, CstR, for CsoR-like sulfurtransferase repressor,[29] regulates the transcription of a sulfide oxidation system that is partly used to clear excess sulfide in *S. aureus* strain Newman.[24]

Here, we show that both H_2S homeostasis and cellular RSS level impact virulence gene regulation in *S. aureus*. We show that the endogenous effector molecules of H_2S signaling, RSS, can be regulated in *S. aureus* cells[24] and that proteome *S*-sulfhydration is widespread, involving both metabolic enzymes and transcriptional regulators. Distinct levels of RSS impact the expression of secreted proteins involved in immune evasion and secretome cytotoxicity to different degrees, due in part to *S*-sulfhydration of global virulence regulators including MgrA. Two previously uncharacterized thioredoxin-like proteins are *S*-sulfhydrated in sulfide-stressed cells, designated TrxP and TrxQ, and these enzymes are characterized by significant depersulfidase activity. We propose on the basis of this and previous work[24] that TrxPQ and thioredoxin reductase (TrxB) comprise a hydrodisulfide reduction system that reversibly controls global proteome *S*-sulfhydration and protects *S. aureus* from the effects of reactive oxygen and reactive nitrogen species.

RESULTS

Chemical Profiling Reveals Significant RSS in *S. aureus*

We previously reported on a ratiometric ($^{34}S/^{32}S$) mass spectrometry-based analytical method to detect and quantify bimane derivatives of endogenous inorganic and organic RSS in wild-type *S. aureus* coincident with induction of the *cst* operon by exogenous Na_2S or Na_2S_4 added to cells.[20,24] Here, we show that these RSS levels can be affected by genetic background and growth conditions. The concentration of the major reducing LMW thiol, bacillithiol (BSH), is in the ≈3 mM range, as expected,[30] with the concentration of coenzyme A (CoASH) and cysteine (CysSH) comparable and ≈3-fold lower, respectively, relative to BSH (Table S1). The corresponding concentrations of endogenous ($t = 0$, prior to addition of 0.2 mM Na_2S to mid log, exponentially growing cells) bacillithiol persulfide (BSSH), cysteine persulfide (CysSSH), and coenzyme A persulfide (CoASSH) are 2.4%, 1.4%, and 1.8% of the total corresponding thiol, respectively, in wild-type cells (Table S1; Figure 1B).

We detect a substantial and nearly uniform ≈20-fold increase in BSSH, CysSSH, and CoASSH (Figure 1) and ≈50-fold increase in inorganic RSS (Figure S1) in *S. aureus* 30 min following addition of 0.2 mM Na_2S to cells, revealing a rapid change in sulfur speciation from inorganic Na_2S to organic LMW thiol persulfide species upon acute-phase sulfide addition. The same is true in the Δ*cstR* and Δ*cysM*/Δ*metB* strains, with these organic RSS significantly lower than the wild-type strain at all time points (Figure 1A), as is the fraction of the thiol species present as persulfides (Figure 1B). These lower RSS in the Δ*cstR* and Δ*cysM*/Δ*metB* strains at $t = 0$ are largely complemented in the Δ*cstR*::CstR and Δ*cysM*/Δ*metB*::CysM/MetB strains, with the possible exception of CoASSH in the Δ*cstR* strain (Figure 1C, D). These data are consistent with the hypothesis that *CstR*-regulated enzymes lower circulating H_2S and help mediate RSS clearance[21] and that CysM and/or MetB generate significant endogenous H_2S (Figure S1) capable of modulating organic RSS levels in *S. aureus*. We note, however, that CysM and MetB cannot be the only contributors to cellular RSS since their deletion reduces organic persulfide levels by just 2–3-fold.

Interestingly, in contrast to exogenous sulfide treatment, addition of exogenous H_2O_2 (0.3 or 10 mM) to wild-type *S. aureus* gives rise to decreases in H_2S, inorganic RSS (Figure S1E),

and organic RSS (Figure S1F). These data are consistent with the reactivity of H_2S toward H_2O_2, a protective effect of organic RSS against ROS stress, and generally opposite transcriptomic profiles associated with RSS and ROS in *S. aureus*.[5,18,24] In any case, the quantitation of cellular RSS reveals that key inorganic and organic H_2S signaling molecules can be controlled endogenously by genetic background or by addition of exogenous sulfide. This allows us to test the impact of perturbation of RSS on specific metabolic and regulatory processes.

Profiling of Proteome *S*-Sulfhydration before and after Acute Sulfide Stress

Protein *S*-sulfhydration is thought to result from oxidation of reactive protein thiols by RSS to create a hydrodisulfide moiety, RSSH, the presumed mechanism by which H_2S signals and impacts specific biological processes.[6,31] Similar to other PTMs, protein *S*-sulfhydration on an active site thiol is likely to alter protein activity while this same modification on a transcriptional regulator could potentially influence gene regulation.[4] To determine if H_2S signals via protein *S*-sulfhydration in *S. aureus* and to identify biological processes that may be influenced by H_2S, we developed a proteome-wide approach to profile protein *S*-sulfhydration, similar to those recently reported for mammalian cells (Figure 2A).[6,31] This protocol leverages the nucleophilicity of the persulfide group[16,32] and incorporates a biotin tagging strategy used to label and enrich lysate sample for peptides containing either reduced or *S*-sulfhydrated cysteine residues, followed by trypsin digestion and immobilization on neutravidin beads. A final reduction step with tris(2-carboxyethyl) phosphine (TCEP) leads to elution specifically of those peptides originally trapped as persulfides. We note however that this method, like others,[6,31] does not detect the persulfide moiety directly like recently described “tag-switch”-based methods,[33,34] and thus may be slightly impacted by the detection of other redox states of cysteine beyond the persulfide.

Three replicate experiments were carried out with early log phase wild-type cells both untreated (N1–N3) and after 20 min of exogenous sulfide (0.2 mM Na_2S; mid log cells) stress (S1–S3). When proteolytic digests of sulfide-stressed cell lysates are analyzed directly by LC-MS/MS (in the absence of the biotin enrichment step), 209 cysteine residue-containing peptides and 15 *S*-sulfhydrated peptides are identified, which corresponds to 7.8% and 0.6% of all detected peptides. The number of cysteine residue-containing peptides increases to ≈38% after the samples are processed by our enrichment method (Figure S2A). The experiments are reproducible, with ≈90% of peptides identified in each experiment occurring at least twice within the three replicates in each condition (Figure S2B). We observe 347 (±24) endogenously *S*-sulfhydrated peptides mapping to 238 (±15) proteins in unstressed, mid log *S. aureus* cells, fully consistent with the readily detectable levels of organic RSS found prior to addition of exogenous sulfide to these cultures (Figure 2B,C). These numbers increase to 468 (±53) peptides mapping to 305 (±21) proteins upon exogenous sulfide stress (Figure 2B,C).

We note that this approach, like others previously reported,[6,31] is not quantitative and provides no information on the percentage of persulfidation of any identified cysteine. It instead simply maps those sites that have some degree of *S*-sulfhydration in one or the other growth condition. However, a semiquantitative analysis of the relative extent of *S*-sulfhydra-

tion of each protein in unstressed (N*i*, where *i* = 1, 2, or 3) vs sulfide-stressed (S*i*) conditions (Figure 2D) shows that, of the 325 proteins that are reproducibly *S*-sulfhydrated in sulfide-stressed cells, 281 are also modified in unstressed cells (Table S2). This is consistent with a reservoir of protein thiols that contribute, alongside LMW thiols, to persulfide speciation in exponentially growing cells. In order to assess the false positive rate for the method, we performed negative control experiments in triplicate by chemically reducing the cell lysate with TCEP prior to biotin tagging and adsorption to the neutravidin beads. Since treatment with TCEP should result in all cysteine residue side chains being reduced, they should all be subsequently tagged by biotin in a form that does not allow for final elution from the column under reducing conditions. Consistent with this, we detect cysteine residue-containing peptides from just 16 proteins that appear in at least two of these three replicate negative control experiments which are also detected reproducibly to be *S*-sulfhydrated in cells (Table S2). This suggests that *S*-sulfhydrated peptides are detected with a false positive rate of ≈5% and are thus detected with ≈95% confidence.

Three classes of *S*-sulfhydrated proteins are obtained on the basis of the $\Sigma Si/(\Sigma Ni + \Sigma Si)$ value: ≈50 proteins are extensively *S*-sulfhydrated only in sulfide-stressed cells ($\Sigma Si/(\Sigma Ni + \Sigma Si) \geq 0.85$), with ≈25 or so under *S*-sulfhydrated in sulfide-stressed cells ($\Sigma Si/(\Sigma Ni + \Sigma Si) \leq 0.35$). The majority of *S*-sulfhydrated proteins cluster around the median $\Sigma Si/(\Sigma Ni + \Sigma Si)$ value (0.62) and include the most highly abundant proteins in the cell, consistent with the significant levels of basal *S*-sulfhydration of the proteome. The $\Sigma Si/(\Sigma Ni + \Sigma Si)$ value is a reflection of *S*-sulfhydration levels on a particular protein before and after sulfide addition to cells and is not representative of the general expression levels of that protein, as revealed by a comparison of $\Sigma Si/(\Sigma Ni + \Sigma Si)$ and transcriptomic analysis of 325 reproducibly detected proteins (Figure S2C) before and after sulfide stress.[24] The vast majority (96%) of *S*-sulfhydration targets in Figure 2D show no correlation between $\Sigma Si/(\Sigma Ni + \Sigma Si)$ and change in mRNA level by sulfide stress (Figure S2C); those that do include genes are either strongly repressed (*cysK*; NWMN_0115; NWMN_1951) or derepressed (*cstAB*) by exogenous sulfide treatment.[24]

A bioinformatics analysis reveals that *S*-sulfhydrated proteins can be mapped to distinct metabolic pathways. For example, proteins known to function in sulfur metabolism as cellular carriers of persulfide groups or in LMW thiol dithiol–disulfide exchange, including CstA and CstB in the *cst* operon observed previously *in vitro*,[35,36] CoA disulfide reductase (Cdr), a thiol peroxidase (Tpx), the thioredoxin reductase (TrxB),[37] methionine sulfoxide reductase (MsrB), and a candidate bacillithiol disulfide oxidoreductase (NWMN_1388), are all *S*-sulfhydrated, validating our profiling method. Of special interest are several transcriptional regulators, including the heat shock regulator CtsR that controls the expression of the *clp* protease genes linked to virulence,[38] the alternative sigma factor SigB, a global stress response regulator that regulates the transcription of the global regulator SarA,[39] RsbU, which regulates the expression of SigB,[40] and an uncharacterized dithiol-containing TetR-family repressor, NWMN_2477, and all are detected as *S*-sulfhydrated in cells. Other regulators, including WhiA, which controls sporulation,[41] MraZ, which regulates cell division,[42] and HssR, a heme response regulator,[43] are also *S*-sulfhydrated, suggesting that these regulators may be subject to redox-regulation as potential targets for H_2S signaling.

Strikingly, of the nine known SarA family proteins in *S. aureus* that harbor a single Cys proposed as a potential regulatory site,[44] four, including SarA, SarS, SarR, and MgrA, are *S*-sulfhydrated in both uninduced and sulfide-stressed cells to varying degrees. The identification of this modification on these global virulence regulators is of interest given that a comprehensive chemical understanding of how these virulence regulators are triggered to regulate their global transcriptional profiles remains lacking.[45,46] However, reversible oxidation on the single active cysteine residue of SarA and MgrA has been proposed as a potential mechanism to regulate global virulence gene expression.[46] As a reversible oxidation on cysteine (see below), protein *S*-sulfhydration may also serve a regulatory role.

S-Sulfhydration on MgrA Decreases Its DNA Binding Affinity

To test the regulatory potential of protein *S*-sulfhydration on gene expression, we purified MgrA as representative of a SarA-family repressor that is *S*-sulfhydrated in cells (Figure 2D). MgrA harbors a single redox-sensitive cysteine, Cys12, at the dimer interface, oxidation of which leads to DNA dissociation.[47] Reduced MgrA was *S*-sulfhydrated at Cys12 to ≈40% *in vitro* (see Supporting Experimental Procedures; confirmed by LC-MS/MS, Figure S3C) and the DNA binding affinity to the *ebh* promoter (P_{ebh}) sequence operator[48] compared to that of fully reduced MgrA using an electrophoretic gel mobility shift assay. Compared to unmodified MgrA, MgrA-SSH binds to P_{ebh} with weaker affinity (K_a of $3.0 \pm 0.6 \times 10^8$ M^{-1} for MgrA vs $1.3 \pm 0.2 \times 10^8$ M^{-1} for MgrA-SSH; see Figure S4A), and this diminution in binding affinity, while modest, is reversible by treatment with dithiothreitol (DTT) to obtain rereduced MgrA (Figure 3A). This finding is consistent with the hypothesis that RSS are capable of modifying MgrA activity and perhaps other global regulators harboring redox-sensitive cysteines, thus impacting their transcriptional programs. Indeed, a number of SarA-regulated genes (SarA is also *S*-sulfhydrated in cells; Figure 2D) that are massively up-regulated in postexponential phase, including fibronectin binding protein A (*fnbA*), coagulase (*coa*) and fibrinogen binding protein (*fib*), are all up-regulated by higher levels of RSS (Figure 1) and down-regulated in Δ*cstR* and Δ*cysM*/Δ*metB* strain, characterized by lower levels of RSS (Figure 1; Figure S4B). Since staphylococcal global regulators constitute a highly integrated and complex network featuring both coordinated up- and down-regulation, a simple correlation between *S*-sulfhydration and transcription output is not expected; however, taken collectively, they suggest that *S*-sulfhydration can modulate global expression of virulence factors.

Different Endogenous RSS Levels Differentially Impact the Expression of Secreted Virulence Factors

To determine if perturbation in cellular RSS is capable of impacting extracellular virulence factor protein abundance, we characterized secreted protein (secretome) fractions from three *S. aureus* Newman strains or conditions relative to an untreated wild-type control. These are the wild-type strain subjected to exogenous sulfide stress (0.2 mM Na_2S; WT + S^{2-}), characterized by elevated RSS relative to an untreated wild-type strain (Figure 1; Figure S1), and the Δ*cstR* and Δ*cysM*/ Δ*metB* strains, both of which show lower RSS levels compared to the untreated wild-type strain, albeit for different reasons (Figure 1; Figure S1). All four strains or conditions are characterized by similar growth kinetics (Figure S5A). Samples for secretome analysis taken at postexponential and stationary phases, when extracellular toxins

and enzymes are maximally expressed, were analyzed on a denaturing gel to visualize their protein patterns. The three strains/conditions have readily distinguishable distributions of secretome proteins relative to the untreated wild-type strain (Figure 3B). In addition to the Newman strain, a MRSA strain, FPR3757, was also tested and shows distinct protein patterns among wild-type, wild-type plus sulfide, and a Δ*cysM*/Δ*metB* strain at both postexponential and stationary phases (Figure S5B).

To test if altered secretomes impact cell cytotoxicity, we measured the viability of several different mammalian cell lines following incubation with secretome fractions derived from each of the four *S. aureus* Newman strains/conditions. HEK293T cells are characterized by increased viability (*decreased* killing) when incubated with the secretome derived from sulfide-treated wild-type cells collected at the post-exponential phase but less viable with the secretome fraction derived from the Δ*cysM*/Δ*metB* strain collected at the same growth phase, relative to the secretome obtained from the untreated, wild-type strain (Figure 3C). In addition to HEK293T cells, other cell lines including A549, HEK293, and HL-60 show similar trends in differential viability with the secretome fractions from sulfide-stressed wild-type cells or the Δ*cysM*/Δ*metB* strain relative to the untreated wild-type cells, with the effects typically more pronounced with stationary phase vs postexponential phase secretomes (Figure S6). In contrast to the Δ*cysM*/Δ*metB* strain, we observe no significant impact of the Δ*cstR* strain on mammalian cell viability relative to untreated wild-type cells. This suggests that differential RSS is just one factor that impacts the cytotoxicity of secreted virulence factors used to kill host cells. In fact, when mice are infected with the Δ*cstR* strain relative to the wild-type *S. aureus,* bacterial loads are significantly lower in the kidney in the mutant strain, suggesting that unregulated expression of the *cst*-encoded RSS-clearance genes is detrimental to survival in this niche (Figure 3D).

In an effort to attribute differences in cell killing to specific secretome proteins, the four secretome fractions were subjected to label-free quantitative $HDMS^E$ analysis.[49] If RSS impacts virulence regulation, the concentrations of virulence factors in the secretome derived from sulfide-stressed wild-type cells and the two deletion strains may well change in opposite ways relative to the untreated wild-type control. Of the 41 extracellular virulence proteins detected (Figure S7), 11 show a statistically significant difference ($p < 0.05$) between the sulfide-stressed wild-type secretome relative to the two deletion strains. Six are encoded by the superantigen-like (*sslnm*) protein operon (NWMN_0388-0397), and five are direct or indirect targets of MgrA regulation (Figure 3E). The decreased abundance of the SSLs and FPRL1 inhibitory protein in the Δ*cstR* strain and the secretory antigen SsaA (NWMN_2199) under sulfide-stressed conditions are all consistent with transcriptomic experiments.[24] Interestingly, α-hemolysin and phenol soluble modulin α4 peptide, as the only toxins among these 11 proteins to directly mediate cell killing, are present at lower levels in sulfide-stressed secretome, which tracks with the differences in secretome cytotoxicity (Figure 3E). These findings suggest that H_2S homeostasis may be linked to virulence factor expression via *S*-sulfhydration of global regulators.

S-Sulfhydration of the Active Site Cysteine of Glyceraldehyde-3-Phosphate Dehydrogenase Negatively Impacts Enzyme Activity

Previous work reveals that enzyme *S*-sulfhydration affects specific activities leading to inactivation or activation, thus impacting a number of biological processes including carbohydrate metabolism.[13–15,50,51] Indeed, the activity of mammalian glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been reported to be augmented[12] or inhibited[52] by *S*-sulfhydration on the catalytic cysteine, while *S*-sulfhydration of protein tyrosine phosphatase was found to be reversibly inactivated by H_2S-induced sulfhydration of the active-site cysteine.[53]

We identified a number of metabolic enzymes as *S*-sulfhydrated in *S. aureus* cells including the active site thiol, C151, of GAPDH (GapA) (Figure 2D; Table S2). To test if *S*-sulfhydration has an effect on specific activity, we incubated purified GAPDH with polysulfides and obtained partially derivatized (≈53%) GAPDH *S*-sulfhydrated at C151, confirmed by LC-MS/MS (Figure S3A), and measured its specific activity. This preparation of *S*-sulfhydrated GapA (GapA-SSH) is ≈25% less active with no effect on the K_m (Figure 4A), with the kinetic impact of active-site *S*-sulfhydration on GapA reversible by DTT treatment (Figure 4B). Thus, GAPDH *S*-sulfhydration is clearly inhibitory. In addition, a large number of proteome nonconserved and nonactive-site cysteines were found to be *S*-sulfhydrated in cells (Figure S8). These include Cys71 of lactate dehydrogenase 2 (Ldh2), which is readily modified both *in vivo* (Table S2; Figure 2D) and *in vitro* (Table S4), resulting in a stimulation of Ldh2 activity (Figure S8E). The biological implications of these modifications are unknown but would potentially provide a cellular reservoir of bioactive sulfur.

Two Thioredoxin-Like Proteins Catalyze Protein *S*-Sulfhydration Reduction

In order for proteome *S*-sulfhydration to be regulatory, a catalytic system must be present to remove proteome hydrodisulfides deposited there by cellular RSS. Recent findings reveal that the major thioredoxin in mammalian cells, Trx, is also capable of reducing protein hydrodisulfides (persulfides) in addition to its well-established activity on protein disulfides.[31,33] Strikingly, two uncharacterized thioredoxin-like proteins encoded by locus tags NWMN_0774 (of known structure; see Figure 5) and NWMN_0779 were found to be *S*-sulfhydrated only in sulfide-stressed cells and are therefore excellent candidates as protein "depersulfidases" (Figure 2D; Table S2), as described previously for the mammalian thioredoxin (Trx).[31,33] We find that NWMN_0774 and NWMN_0779 are both capable of reducing *S*-sulfhydrated $CstA^{Rhod}$ (Figure S3D), corresponding to the N-terminal rhodanese domain of the sulfurtransferase CstA[35] as a model substrate, in the presence of authentic thioredoxin reductase, TrxB, and a reductant (Figure 6A; Table S3). We therefore designate NWMN_0774 and NWMN_0779 as *trxP* and *trxQ* (for thioredoxin depersulfidases P and Q), respectively. In addition to $CstA^{Rhod}$, we also tested the depersulfidase activity of TrxP and TrxQ on *S*-sulfhydrated Ldh2 which is modified at Cys71 nearly exclusively in sulfide-stressed cells and *S*-sulfhydrated to ≈70% upon reaction with Na_2S_4[35] *in vitro*. *S*-Sulfhydrated Ldh2 is efficiently reduced by the TrxP–TrxB pair, concomitant with the production of H_2S (Figure 6E,F, Table S4). The *trxP trxQ* double mutant strain exhibits a

growth lag after acute sulfide stress relative to the wild-type strain, consistent with a role in counteracting proteome *S*-sulfhydration under these conditions (Figure 6D).

It is interesting to point out that the authentic thioredoxin of *S. aureus*, TrxA, was not detected in our *S*-sulfhydration profiling experiments (Figure 2D; Table S2). We therefore tested if the thioredoxin homologues in *S. aureus* strain Newman differentially catalyze protein persulfide vs protein disulfide reduction at saturating TrxB and NADPH. We find that TrxA has ≈100-fold higher protein disulfide reductase activity relative to TrxP and TrxQ, with the specific activities of the three thioredoxins toward protein persulfides much closer to one another, within a factor of ≈5 (Figure 6A–C). These data reveal that TrxP and TrxQ are significant protein depersulfidases, particularly under conditions of high cellular RSS, while TrxA preferentially functions to reduce protein disulfide bonds. We propose that TrxP, TrxQ, and TrxB, whose expression is also regulated by SarA,[37] comprise a hydro-disulfide reduction system that controls the levels of global proteome *S*-sulfhydration in *S. aureus*.

DISCUSSION

In this study, we present multiple lines of evidence that link sulfide homeostasis (Figure 7A) and H_2S signaling via low molecular weight RSS and proteome *S*-sulfhydration to global virulence regulation in *S. aureus* (Figure 7B). We have quantified the concentrations of endogenous LMW thiol persulfides and inorganic polysulfides as the effector molecules of H_2S signaling in *S. aureus*. Global proteome *S*-sulfhydration profiling reveals, for the first time, proteins that may be affected by H_2S signaling in a bacterium. *S*-Sulfydration is a PTM that drives changes in enzyme activity and transcriptional regulation, while also providing a potential reservoir of bioactive sulfur as cysteine persulfides on nonconserved and nonessential, solvent-exposed cysteines (Figure S8). This reservoir is potentially tapped by two thioredoxin-like proteins, TrxP and TrxQ, that regulate global proteome *S*-sulfhydration levels in cells as a means to control RSS homeostasis (Figure 7). Perhaps most importantly, we find that the relative abundance of extracellular virulence (secretome) proteins is influenced by cellular RSS levels and that *S*-sulfhydration of cysteine-containing global regulators, including MgrA, may be partly responsible for this.

The physiological importance of sulfide homeostasis and RSS in heterotrophic bacteria, in particular bacterial pathogens, is not well understood. This contrasts with sulfide metabolism in the mitochondria of higher organisms[19] which is known to function in H_2S detoxification via sulfur oxidation, while in sulfide-oxidizing chemolithotrophic[54] and phototrophic[22] bacteria, sulfide is exploited as a source of reducing power to drive energy production and photosynthesis. H_2S and endogenously synthesized nitric oxide have been reported to function synergistically to protect diverse bacteria, including *E. coli* and *S. aureus*, against the effects of oxidative stress induced by common antibiotics via up-regulation of H_2S biogenesis.[7] Recent studies of the mechanism of this protection in *E. coli* suggest that strong oxidants, e.g., hydrogen peroxide, increase cellular H_2S production, leading to excess metabolically derived sulfide. Excess sulfide functions to chelate Fe^{II} under conditions of ROS, thus inhibiting Fe^{II} mediated Fenton chemistry and hydroxyl radical-mediated chromosomal DNA damage.[8] The degree to which this mechanistic scenario is broadly

operative in other bacteria is unclear. We show here that H_2O_2 actually depletes cellular RSS (Figure S1), and increased exogenous sulfide in *S. aureus* transcriptionally induces a Zn and Mn starvation response, with little or no effect on the Fe-Fur regulon.[24] In contrast, exogenous addition of nitroxyl (HNO), a major product of the interplay of RSS and nitric oxide,[55] perturbs both cellular Fe and S speciation with a clear increase in organic RSS, with the transcriptomic data most consistent with an Fe-overload and ROS response.[24]

We suggest on the basis of this work that sufficient cellular RSS influences a dynamic reservoir of proteome *S*-sulfhydration and LMW persulfides in *S. aureus* that function to protect reactive thiols from the effects of irreversible inactivation by oxidative stress.[5,6] Hydrogen peroxide readily sulfenylates (*S*-hydroxylates) reactive cysteine residues[56] thus making the sulfur atom sufficiently electrophilic, allowing for direct attack by H_2S,[32] and like RSS itself (Figure 7B), driving proteome *S*-sulfhydration. This reaction is similar to *S*-thiolation by LMW thiols, known to protect cysteine from higher oxidation states under oxidative and nitrosative stress.[57,58] Analogous chemistry has been proposed for the reaction of *S*-nitrosothiols, formed under conditions of nitrosative stress, with H_2S to create *S*-sulfhydrated thiols with the release of HNO. HNO, like RSS, is a significant inducer of the *cst* operon.[24] We favor the hypothesis that *cst*-encoded enzymes up-regulate H_2S and RSS clearance in order to restore sulfide homeostasis (Figure 7A) by forming more oxidized and less toxic forms of sulfur as a means to tap these beneficial, protective roles of H_2S. Consistent with this, we find significant overlap between those proteins identified as sulfenylated under exogenous H_2O_2 stress[59] and *S*-sulfhydrated here, accounting for ≈50% and ≈ 30% of the proteins in each group, respectively (Figure S2D). This overlap might be expected given the large number of abundant, housekeeping proteins that are *S*-sulfhydration targets and that both modifications will tend to occur on protein thiols with pK_a values lower than H_2S.[34,60] This potentially explains why protein *S*-sulfhydration is protective against ROS and may well be associated in part with the observed virulence defect in mice (Figure 3).

Finally, four SarA family virulence regulators known to be expressed in early to mid log *S. aureus* cultures, SarA, SarR, SarS, and MgrA,[61] are *S*-sulfhydrated but to varying degrees before and after sulfide treatment (Figure 2D). This modification on MgrA negatively regulates DNA binding, suggestive of a redox-switching, or more generally, post-translational modification-based mechanism(s) to control expression of SarA-family regulons. RSS levels clearly impact exoprotein expression, especially of the superantigen-like proteins (SSLs), which are induced by high levels of sulfide and suppressed when RSS levels are low. These findings are consistent with our transcriptomic analysis of the expression of *sslnm* genes, which are strongly repressed in the Δ*cstR* strain.[24] Many SSLs and other superantigens share a common OB-fold/*β*-grasp architecture that harbor protein interaction and/or glycan-binding sites and play significant roles in innate immune evasion by inhibiting neutrophil extravasion and chemokine receptor activation (SSL5), complement activation (SSL7), or fibronection binding (SSL8).[1] However, the transcriptional regulation of *ssl* genes has not been extensively studied and may be regulated by global virulence regulators and/or other forms of stress, e.g., iron starvation.[62] For example, the transcription of *ssl5nm* and *ssl8nm* are up-regulated by the two-component system SaeRS and down-regulated by quorum sensing repressor Agr in the Newman strain,[63] while the toxin

repressor Rot and SaeRS activate the expression of SSLs cooperatively.[64] Further elucidation of the mechanistic connections between RSS signaling, virulence factor regulation, and metabolic regulation in this important human pathogen may well lead to the identification of new antibiotic targets specific to bacterial sulfide homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Work presented in this manuscript was supported by Grants R01 GM097225 and R35 GM118157 (to D.P.G.), R01 AI073843 and R01 AI069233 (to E.P.S.), and T32 HL094296 and F32 F32AI122516 (to L.D.P.) from the US National Institutes of Health. We thank the late Prof. Richard Armstrong, Dr. Mary E. Keithly (Vanderbilt University), and Vanderbilt Center for Chemical Biology for the gift of bacillithiol disulfide used in these studies (supported by NIH R01 GM030910).

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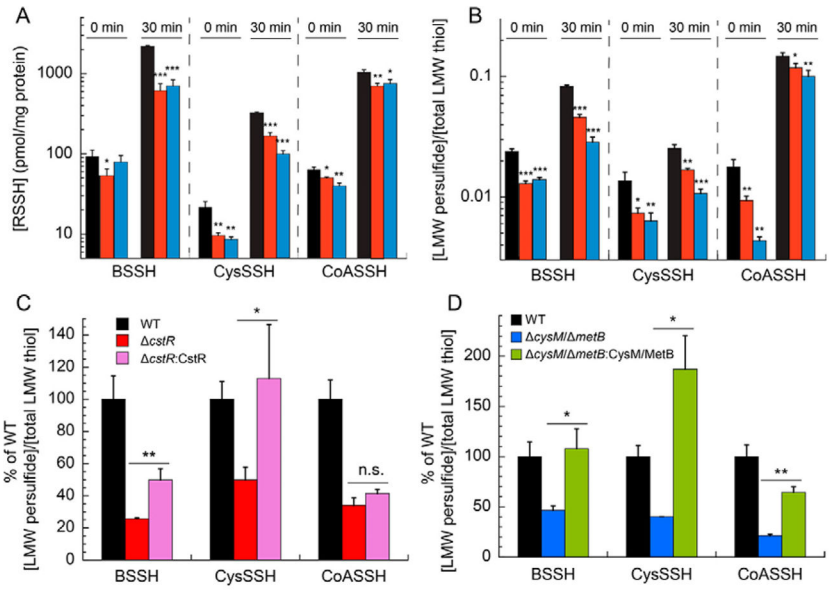


Figure 1.

Quantitation of LMW thiol persulfides in *S. aureus*. (A) Cellular levels of bacillithiol persulfide (BSSH), cysteine persulfide (CysSSH), and coenzyme A persulfide (CoASSH) in *S. aureus* wild-type (black bar), Δ*cstR* (red bar), and Δ*cysM*/Δ*metB* (blue bar) strains measured before (0 min) and 30 min after addition of 0.2 mM sulfide to cultures. (B) In addition to cellular LMW persulfide levels, the ratio of LMW persulfide to total thiol is also plotted in the same way. (C) The percentage of the ratio of Δ*cstR* strain and the complementation strain (Δ*cstR*:CstR, pink bar) at 0 min relative to wild-type. (D) The percentage of the ratio of Δ*cysM*/Δ*metB* strain and the complementation strain (Δ*cysM*/Δ*metB*:CysM/MetB, green bar) at 0 min relative to wild-type. Error bars represent SDs of $n = 3$ experiments, with statistical significance established using a paired *t*-test relative to wild-type strain under the same condition (***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$, n.s., no significant difference).

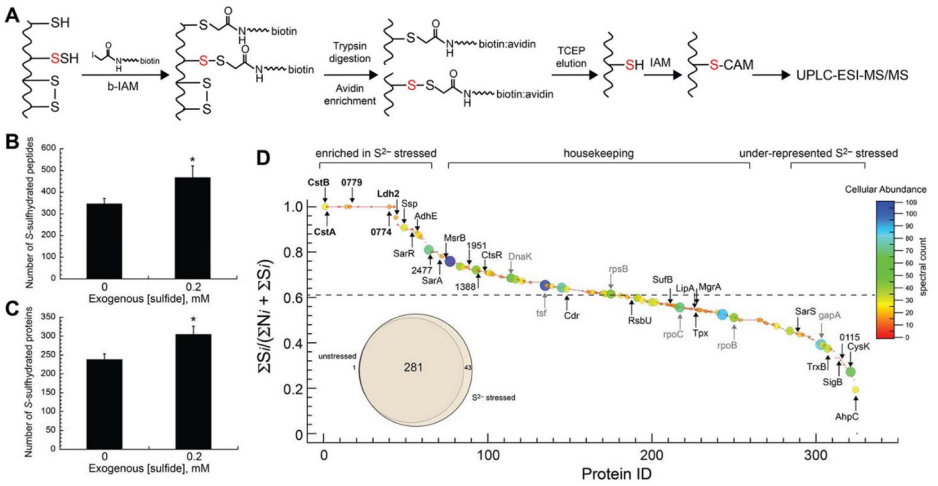


Figure 2.

Enrichment strategy for *S*-sulfhydrated cysteine-containing peptides. (A) Scheme of the strategy used to identify *S*-sulfhydrated peptides and proteins before (0 mM sulfide) or after addition of sulfide (0.2 mM) to cells. The method takes advantage of similar reactivities of thiols vs persulfides toward the iodoacetamides,[35] with both moieties capped with biotinylated iodoacetamide (b-IAM), enriched by neutravidin capture, which, following a washing protocol, is treated with the reductant TCEP, followed by capping eluted peptides with IAM, with carbamidomethylated (CAM) peptides identified by UPLC-ESI-MS/MS. Three biological replicates (i = 1–3) of untreated (Ni) and sulfide-treated (Si) were analyzed in this way (Figure S4), with the mean and standard deviation shown for the number of *S*-sulfhydrated peptides (B) and corresponding proteins (C) enriched by this method. (D) Plot of $\Sigma S_i/(\Sigma N_i + \Sigma S_i)$ (range 0–1), where ΣS_i is the sum of the spectral counts of all CAM-modified peptides in a single protein in all three S experiments and ΣN_i is the sum of the spectral counts of all CAM-modified peptides from that same protein in all three N experiments vs protein ID, arbitrarily arranged from left to right, according to the $\Sigma S_i/(\Sigma N_i + \Sigma S_i)$ (primary sort) and ΣS_i (secondary sort) values. Each symbol corresponds to a single protein (325 total) and is colored and sized according to the approximate abundance of that peptide in sulfide-treated cells, estimated from a sum of the spectral counts for all detectable peptides for a given protein in a single experiment without enrichment (see scale bar).

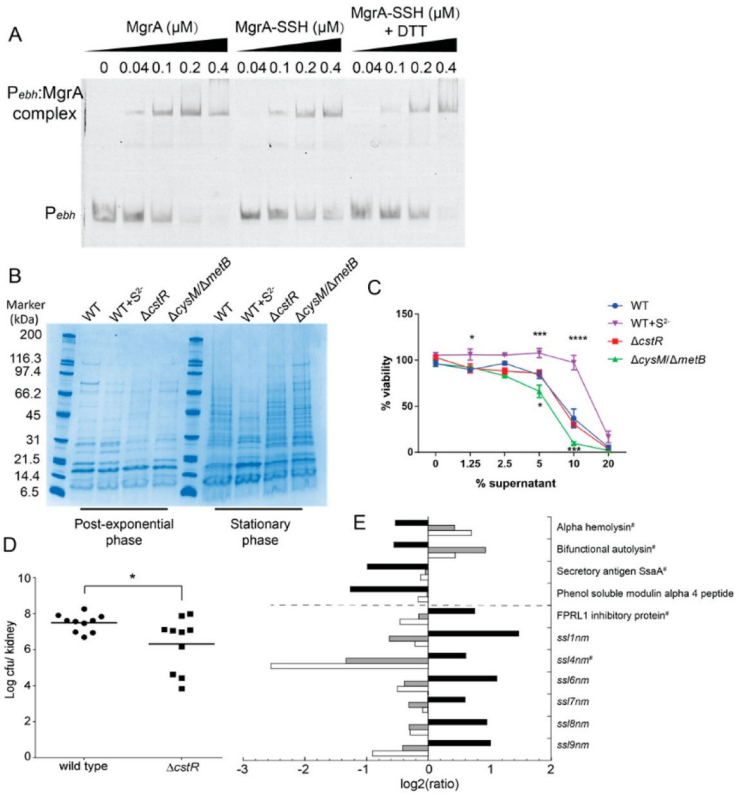
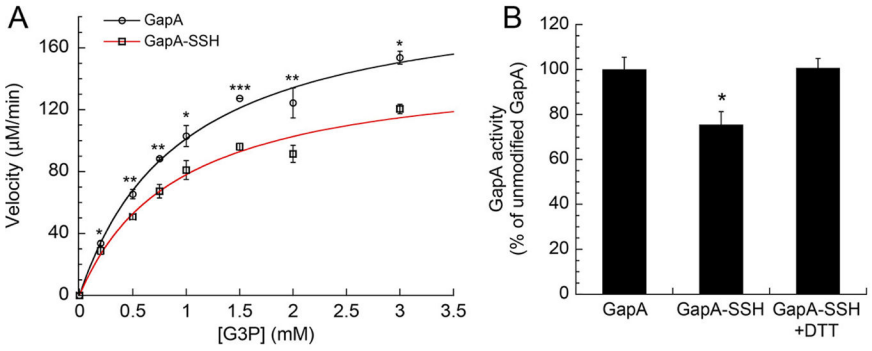


Figure 3.

H_2S signaling affects virulence expression. (A) Electrophoretic mobility shift assay (EMSA) shows that protein *S*-sulfhydration changes the DNA binding affinity of global virulence regulator MgrA to *ebh* promoter. Protein with different concentrations was incubated with 20 nM fluorescein-labeled DNA oligo (P_{ebh}) before separation on native polyacrylamide gel. Unbound P_{ebh} and P_{ebh}/MgrA complex are indicated. (B) The pattern of secreted proteins is shown on SDS-PAGE stained with Coomassie blue. The four lanes under postexponential phase and stationary phase from left to right are WT *S. aureus* under normal growth conditions, WT with sulfide stress, Δ*cstR*, and Δ*cysM*/Δ*metB* strains. The remaining two lanes are protein markers. The black triangle pointed band is likely to be an ensemble of superantigen-like proteins, which is checked by LC-MS/MS of the gel band. (C) The secretome sample was prepared by concentrating the supernatant after the culture was centrifuged. The cytotoxicity of the secretome of different strains at postexponential phase was tested by measuring the viability of HEK293T cell line incubated with different dilutions of secretome sample for 22–24 h. The % viability is compared to the mean of the wells with only medium added ($n = 40$). The mean of 2–4 technical replicates was then graphed as mean ± SEM. Statistics are compared to WT at each time point by two-way ANOVA with Dunnett's multiple comparisons adjustment (*$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$). (D) The Δ*cstR* strain is less virulent than the wild-type strain. Balb/c mice with retroorbitally infected with 1.7×10^7 and 2.1×10^7 cfu (colony-forming units) of wild-type and Δ*cstR* strains, with the infection allowed to proceed for 96 h before the mice were sacrificed, organs harvested, and cfu in the kidney determined by homogenization and plating of serial dilutions. Horizontal bars, mean cfu; *, *p*-value ≤0.05. (E) Expression levels

of secreted proteins in WT *S. aureus* with sulfide stress, Δ*cstR*, and Δ*cysM*/Δ*metB* strains compared to WT under normal growth conditions. Black bars mean the ratio of protein levels in WT with sulfide stress over WT under normal growth conditions. Gray bars mean the ratio of Δ*cstR* strain over WT under normal growth conditions. White bars mean the ratio of Δ*cysM*/Δ*metB* strain over WT under normal growth conditions. Pound sign (#) labeled proteins are direct or indirect targets of MgrA regulation.



C

	K_m (mM)	V_{max} (μM/min)	k_{cat} (×10^3 min^{-1})	k_{cat}/K_m (×10^6 M^{-1} min^{-1})
GapA	1.0 ±0.1	199 ±11	4.0 ±0.2	4.1 ±0.6
GapA-SSH	0.9 ±0.2	150 ±11	3.0 ±0.2	3.3 ±0.7

Figure 4.

Reversible protein *S*-sulfhydration on GapA decreases its enzyme activity *in vitro*. (A) Enzyme activity of GapA and GapA-SSH using glyceraldehyde 3-phosphate (G3P) as the substrate, with each data set fitted to the Michaelis–Menten model. (B) Enzyme activities of GapA-SSH and GapA-SSH with DTT treatment compared to that of GapA. (C) Calculated enzyme catalysis parameters of GapA and GapA-SSH. The experiments were conducted in three replicates with SD represented by error bars. Statistical analysis was established by a paired *t*-test relative to the activity of GapA (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

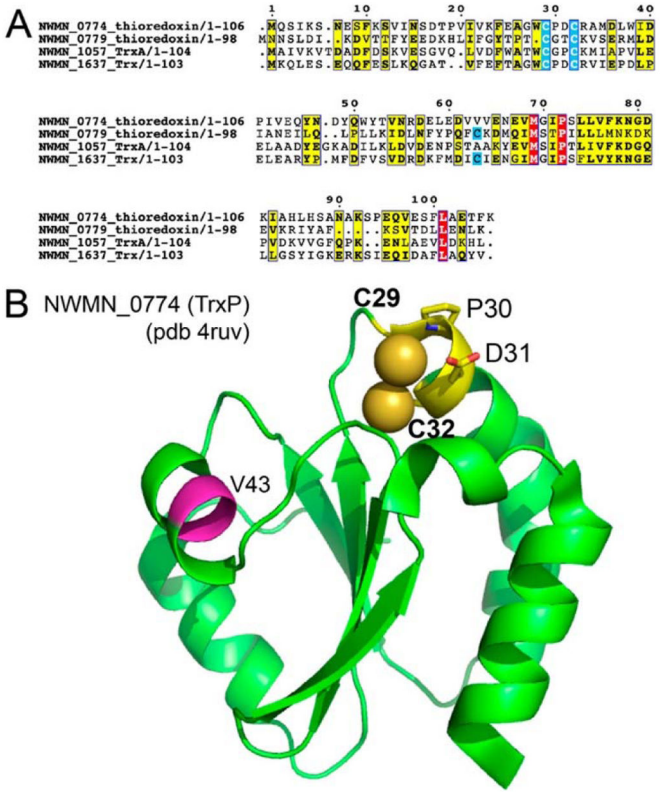


Figure 5.

S. aureus strain Newman encodes for four thioredoxin-like proteins. (A) Multiple sequence alignment of *S. aureus* strain Newman thioredoxin- and thioredoxin-like proteins. The alignment compares the sequences of authentic thioredoxin (TrxA) with two *S*-sulfhydrated thioredoxin-like proteins purified and characterized here, NWMN_0774 (TrxP) and NWMN_0779 (TrxQ), and NWMN_1637, not yet characterized. (B) The X-ray structure of oxidized TrxP, with the active site **C**PD**C** region highlighted (C29 and C32 are *S*-sulfhydrated in cells; Table S2) and V43, the approximate position of the third Cys in TrxQ (of unknown structure) which is *S*-sulfhydrated in cells (Table S2).

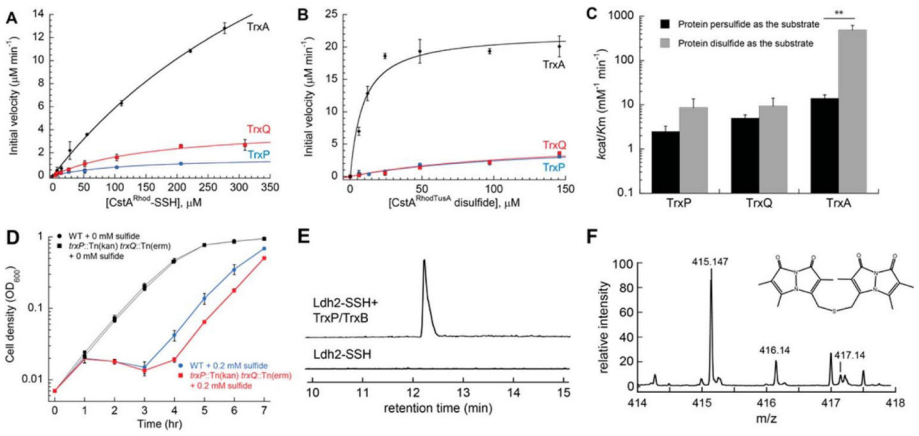


Figure 6.

NWMN_0774 and NWMN_0779 encode thioredoxin-like proteins that are capable of reducing protein persulfides *in vitro*. Steady-state kinetic analysis of NWMN_0774 (TrxP), NWMN_0779 (TrxQ), and TrxA as protein ($CstA^{Rhod}$) persulfide reductases (A) and protein ($CstA^{RhodTusA}$) disulfide reductase (B) in the presence of TrxB and NADPH. Enzyme parameters are summarized in Table S3. (C) Comparison of the catalytic efficiency of TrxP, TrxQ, and TrxA to reduce protein persulfide and protein disulfide. Statistics are established on each enzyme by comparing the activity toward different substrates (**$p < 0.01$). (D) Growth curves of wild-type *S. aureus* and the double mutant strain of TrxP and TrxQ (*trxP*::Tn(kan) *trxQ*::Tn(erm)) under normal growth conditions and sulfide stress conditions. (E, F) Detection of H_2S as a product of Ldh2-SSH reduction by TrxP and TrxB. (E) LC chromatogram of mBBr-labeled H_2S following treatment with TrxP/TrxB, compared to the nonreduced sample. (F) MS identification of the peak at 12.2 min (panel D) as mBBr-labeled H_2S (m/z 415.14 $[M + H]^+$),[65] with the structure shown in the inset.

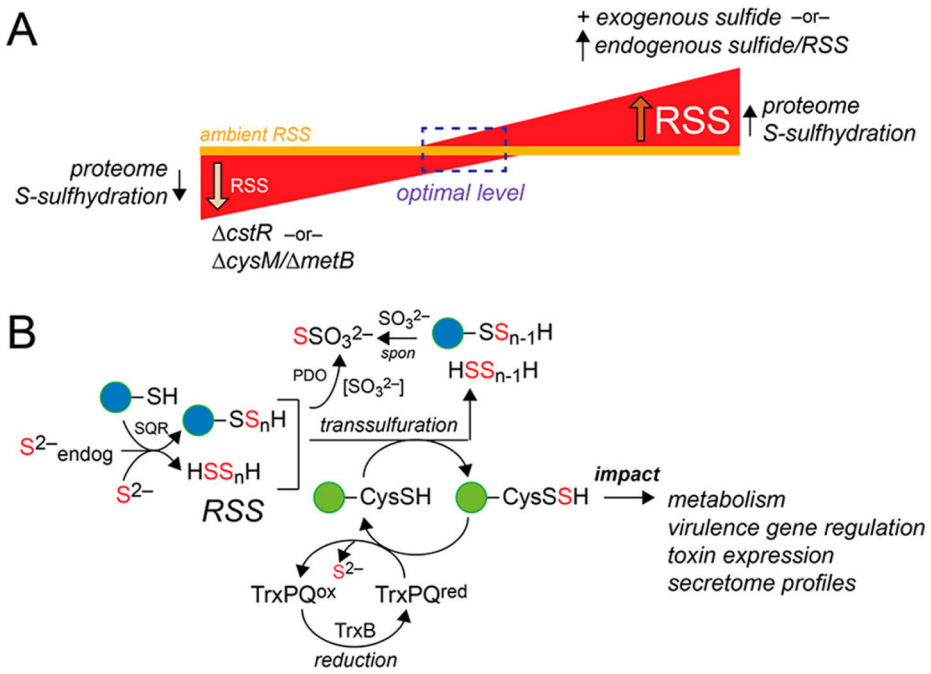


Figure 7.

(A) A model of sulfide homeostasis and (B) a summary of the flow of sulfane sulfur studied here. This cartoon representation of sulfide homeostasis posits that there is an optimal level of cellular RSS that can be perturbed by exogenous sulfide or increases in endogenous RSS, thus resulting in increased proteome *S*-sulfhydration, or decreased by increasing RSS clearance (in the Δ*cstR* strain) or lowering H_2S biogenesis (as in the Δ*cysM*/Δ*metB* strain). (B) Downstream effects of increased endogenous cellular S^{2-}. RSS are derived from the activity of a type II sulfide–quinone oxidoreductase (SQR) and a LMW thiol (blue sphere),[20] which can be oxidized to thiosulfate by a persulfide dioxygenase (PDO),[36,66] spontaneously decay in the presence of sulfite (spon),[67] or engage in transsulfuration (persulfide transfer) reactions[68] with the other small molecules or proteome thiols (green sphere). *S*-Sulfhydration of global virulence regulators can potentially impact a number of different processes including virulence gene regulation, toxin expression,[24] and the nature of secretome profiles. Proteome *S*-sulfhydration is reversed by TrxP or TrxQ in collaboration with the thioredoxin reductase, TrxB.