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Hydrogen Sulfide Sensing through Reactive Sulfur Species (RSS) and Nitroxyl (HNO) in *Enterococcus faecalis*

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Abstract

Recent studies of hydrogen sulfide (H₂S) signaling implicate low molecular weight (LMW) thiol persulfides and other reactive sulfur species (RSS) as signaling effectors. Here, we show that a CstR protein from the human pathogen *Enterococcus faecalis* (*E. faecalis*), previously identified in *Staphylococcus aureus* (*S. aureus*), is an RSS-sensing repressor that transcriptionally regulates a *cst*-like operon in response to both exogenous sulfide stress and Angeli's salt, a precursor of nitroxyl (HNO). *E. faecalis* CstR reacts with coenzyme A persulfide (CoASSH) to form interprotomer disulfide and trisulfide bridges between C32 and C61', which negatively regulate DNA binding to a consensus CstR DNA operator. A *cstR* strain exhibits deficiency in catheter colonization in a catheter-associated urinary tract infection (CAUTI) mouse model, suggesting sulfide regulation and homeostasis is critical for pathogenicity. Cellular polysulfide metabolite profiling of sodium sulfide-stressed *E. faecalis* confirms an increase in both inorganic polysulfides and LMW thiols and persulfides sensed by CstR. The *cst*-like operon encodes two authentic thiosulfate sulfurtransferases and an enzyme we characterize here as an NADH and FAD-dependent coenzyme A (CoA) persulfide reductase (CoAPR) that harbors an N-terminal CoA

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Notes

The authors declare no competing financial interest.

Supporting Information

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Supporting Tables S1–S3, supporting Figures S1–S10, and a detailed description of the experimental materials and methods used in this study (PDF)

disulfide reductase (CDR) domain and a C-terminal rhodanese homology domain (RHD). Both cysteines in the CDR (C42) and RHD (C508) domains are required for CoAPR activity and complementation of a sulfide-induced growth phenotype of a *S. aureus* strain lacking *cstB*, encoding a nonheme Fe^{II} persulfide dioxygenase. We propose that *S. aureus* CstB and *E. faecalis* CoAPR employ orthogonal chemistries to lower CoASSH that accumulates under conditions of cellular sulfide toxicity and signaling.

Graphical Abstract

Emerging studies suggest that hydrogen sulfide (H₂S) and/or H₂S-derived reactive sulfur species (RSS) play beneficial roles as a signaling molecule in mammalian systems,¹ and contribute to the cellular response to reactive nitrogen species (RNS), reactive oxygen species (ROS),^{2–7} and antibiotics stress.^{8,9} These growth-promoting functions of RSS are balanced by a potent inhibition by H₂S of heme-containing enzymes and cellular respiration through poisoning of cytochrome c oxidase¹⁰ and the ability to precipitate transition metals into insoluble complexes.¹¹ H₂S can freely pass through biological membranes, existing mainly as hydrosulfide anion (HS[–]) once inside the cell at physiological pH.¹² H₂S can also be endogenously produced by enzymes of reverse trans-sulfuration and cysteine degradation pathways, extensively studied in mammalian systems, and include cystathionine β -synthase (CBS), cystathionine γ -lyase (CSE), 3-mercaptopyruvate sulfurtransferase (3-MST), and D-amino acid oxidase (DAO).^{13,14} Considering both endogenous and exogenous production of H₂S and H₂S-derived RSS in living systems as well as myriad beneficial and deleterious roles, efficient regulatory strategies that control cellular H₂S concentrations and speciation are required. In certain organisms, excess H₂S is effluxed via a specific hydrosulfide ion channel,¹⁵ whereas other organisms chemically assimilate H₂S using a sulfide oxidation system.¹³

We previously characterized the *cst* operon from the major human pathogen *Staphylococcus aureus* (*S. aureus*).¹⁶ The *cst* operon is transcriptionally regulated by the repressor, CstR, which responds to cellular sulfide stress through sensing low molecular weight (LMW) persulfides and inorganic polysulfides (S_n^{2–}) collectively termed RSS.¹⁶ LMW persulfides can be produced enzymatically in a number of ways, including the oxidation of S^{2–} by

sulfide:quinone oxidoreductase (SQR) and a LMW thiol,^{17,18} *via* 3-MST,¹⁹ which produces reactive inorganic polysulfides,^{20,21} and *via* cysteinyl tRNA synthetase, which catalyzes the formation of cysteine persulfides in a PLP-dependent fashion that can also be incorporated into proteins translationally.²² Nonenzymatic LMW persulfide production, on the other hand, can result from the reaction of sulfide with LMW disulfides, attack of inorganic dihydropolysulfides (HS_nH , $n \geq 2$) or organic polysulfides (RS_nH , $n \geq 2$) on sulfenylated (*S*-hydroxylated) thiols,⁶ or reaction of inorganic polysulfides with organic thiols.¹⁹ Recent work establishes that H_2S and nitric oxide (NO) also react to form a number of bioactive species, including highly reactive polysulfides that activate TRPA1 channels,²³ LMW persulfides,⁷ and the one-electron reduced form of the radical $\text{NO}^\bullet$, nitroxyl (HNO), which is a potent, thiophilic electrophile.^{3-5,7,24,25}

These processes collectively create a pool of labile sulfane (sulfur-bonded) sulfur found in LMW persulfides, which can accumulate to significant levels in both mammalian and bacterial cells,²⁶ with CBS and CSE as significant sources of endogenous H_2S .^{2,27} LMW persulfides, as well as significant proteome sulfuration (also referred to as *S*-sulfhydration or persulfuration²⁸) documented to occur in both mammalian and bacterial cells,^{26,27,29,30} are consistent with emerging evidence to suggest that RSS may be protective against irreversible and inactivating oxidative and electrophilic modifications³¹ and may also impact the extent of proteome nitrosation.³² Indeed, coupling of endogenous H_2S and NO is reported to protect diverse bacterial pathogens, including *S. aureus* against general microbial stress induced by antibiotics, the mechanism of which remains under investigation.^{8,9,33,34} How mammalian and bacterial cells reductively control RSS accumulation and proteome sulfuration is not fully understood, but recent studies implicate thioredoxins and thioredoxin-like proteins and glutaredoxins in this process.^{26,30}

In this work, we present a functional characterization of CstR from a second major human pathogen, *Enterococcus faecalis* (*E. faecalis* or *Ef*), that is also conserved in highly pathogenic strains of *Bacillus anthracis* (*B. anthracis*; Figure 1). The genomic region adjacent to *cstR* suggests a divergently transcribed *cst*-like operon, encoding CstR, two putative sulfurtransferases, RhdA and RhdB, and what we hypothesize is a coenzyme A (CoA) persulfide reductase, CoAPR, encoded by a gene we designate *coaP* (Figure 1). A closely related CoA disulfide reductase-rhodanese fusion protein (CDR-RHD) has been structurally characterized in *B. anthracis* str. Ames alongside the authentic CoA disulfide reductase (CDR)^{35,36} prior to the recognition that this operon might be regulated by RSS.³⁷ Here, we confirm various predictions of this regulatory model and further show that unregulated expression of the *cst*-like operon in a *cstR* *E. faecalis* strain inhibits catheter colonization in a catheter-associated urinary tract infection (CAUTI) mouse model. We provide evidence that *cst*-encoded gene products in both *S. aureus* and *E. faecalis* underpin a common physiological strategy to limit the accumulation of CoASSH and perhaps other RNS-derived CoA adducts^{38,39} under conditions of sulfide stress and/or H_2S /NO cross-talk, which may be particularly important in the urinary tract of infected animals.²⁶

RESULTS

Identification of *cst*-like Operons in *E. faecalis* and *B. anthracis*

In an effort to further understand bacterial sulfide homeostasis and detoxification beyond *S. aureus*, we identified a candidate CstR in *E. faecalis* strain OG1RF, denoted *EfCstR*, which is conserved in all sequenced *E. faecalis* strains, as well as in pathogenic *B. anthracis* species (Figure 1a). Although distinct from the sulfide oxidation system in *S. aureus*, this genomic region is hypothesized to encode proteins involved in sulfur trafficking, including two candidate single-domain sulfurtransferases (rhodanases),⁴⁰ denoted RhdA (OG1RF_RS11625) and RhdB (OG1RF_RS11635), and an enzyme annotated as a CDR-RHD,³⁶ which we propose here is a *CoA* persulfide reductase (denoted *coaP*, encoding CoAPR; OG1RF_RS11630; Figure 1a).⁴¹ In the single *B. anthracis* strain shown, this core *cst* operon region appears to be significantly expanded to include other known cellular persulfide carrier proteins involved in sulfur shuttling and H₂S resistance previously characterized in *S. aureus* (Figure 1a).^{16,26,37,42} Given the consensus GC-rich CstR operator, 5'-ATA|C₄G/CxxxG₂|TAT,⁴³ found upstream of *rhdB* (Figure 1b), we hypothesized that this *cst*-like operon is inducible by H₂S with encoded proteins comprising a novel bacterial strategy for the clearance of cellular RSS.

The *Ef cst*-like Operon Is Transcriptionally Induced by Na₂S and Angeli's Salt

Using quantitative real-time PCR (qRT-PCR), we first showed CstR functions as a repressor since the *coaP*, *rhdA*, and *rhdB* genes are strongly expressed in *cstR* mutant strain relative to an uninduced wild-type strain (Figure 2a and Table S1). As anticipated from earlier work in *S. aureus*,¹⁶ the addition of 0.2 mM Na₂S to early mid log liquid cultures grown microaerophilically results in significant induction of all three genes 10 min postaddition of Na₂S, with comparatively little induction of *cstR* (Figure 2b). The mRNA levels of all three induced genes is decreased by 30 min and further by 60 min, kinetics similar to that observed for sulfide-induction of the *S. aureus cst* operon.¹⁶ This suggests an initial bolus of intracellular sulfide or other downstream RSS is cleared by CstR-regulated gene products and/or *via* one of a number of nonenzymatic mechanisms described above.

Previous studies have shown that endogenously produced H₂S and nitric oxide protect major bacterial species from the effects of antibiotic stress⁸ through a mechanism we have suggested involves the intermediacy of HNO.^{3,5,7,16,24} To investigate the impact of HNO on expression of CstR-regulated genes, we added 0.3 mM Angeli's salt (Na₂N₂O₃), a commonly used precursor of HNO and nitrite (NO₂⁻)⁴⁴ to early mid log cultures grown microaerophilically and monitored *cst* transcription by qRT-PCR.^{44,45} A weaker but significant, acute-phase induction of ~4–15-fold of *coaP*, *rhdA*, and *rhdB* was observed 10 min post-addition of Angeli's salt, with comparatively little induction of *cstR* (Figure 2c), superimposed on a modest growth phenotype (Figure S1c). We observe an analogous induction of the *S. aureus cst* genes by Angeli's salt under aerobic culture conditions, which occurs with a concomitant increase of cellular RSS.⁷ These observations appear to be attributable to HNO since neither sodium nitrite nor an NO[•] donor, diethylamine-NONOate (DEA NONOate), induce the *cst*-like operon nor give a growth phenotype relative to unstressed cells (Figure S1).¹⁶

Profiling of LMW Thiols, Organic, and Inorganic RSS in *E. faecalis* under Sulfide Stress

We next carried out polysulfide metabolite profiling experiments to further elucidate the physiological role of RSS in this process utilizing a recently developed UPLC-ESI-MS/MS method (Figure 3).²⁶ These profiling experiments reveal detectable endogenous H₂S and inorganic polysulfides (Figure 3a), with a ~4–5-fold increase in inorganic sulfide upon the addition of 0.2 mM Na₂S to these microaerophilically grown cultures. These concentrations of H₂S are some ~2-fold (endogenous) to 200-fold (postsulfide addition) lower than in *S. aureus* (Figure 3a).²⁶ H₂S remains consistently elevated 30 min postsulfide addition, while inorganic polysulfides significantly decrease after 90 min over the course of the experiment as the cells enter the late-log phase (Figure S2a). This is likely due to the high reactivity of these inorganic RSS, which are capable of transferring sulfur to LMW thiols present in these cells.¹⁹

Organic RSS profiling experiments (Figure 3b–d) establish that the metabolically important thiol, CoA, is present at ~3000 pmol per milligram of protein in unstressed cells, or ~4 fold higher than cysteine and on the same order of magnitude as glutathione (GSH) and homocysteine (HCySH; Figure 3b). The addition of Na₂S causes a modest increase in LMW thiols of ~2–3 fold by 90 min, increasing further by 150 min (Figures 3b and S2b). The increase in LMW thiols is likely attributed to the fact that cysteine is a metabolic precursor to CoA, HCySH, and GSH as evident by the larger accumulation of these thiols relative to cysteine (Figures 3b and S2b). Cysteine does, however, accumulate ~2-fold by 150 min, which may be due to the fact that H₂S is a substrate for cysteine synthase but is kinetically limited by cellular *O*-acetylserine concentrations.⁴⁶ Strikingly, CoA persulfide (CoASSH) is endogenously present at just ~2 pmol per milligram of protein, which is ~2–5-fold lower than other LMW persulfides (Figure 3c) and ~30-fold lower than in *S. aureus*.²⁶ The addition of Na₂S, however, causes LMW persulfides to increase ~2–3 fold within 30 min. After this time, cysteine persulfide (CysSSH) levels return to approximately endogenous concentrations while all other LMW persulfides are further increased ~3–5 fold (Figures 3c and S2c). Interestingly, CoASSH is the only LMW persulfide to continuously accumulate over the course of the experiment, whereas other LMW persulfides remain relatively constant or decrease after 90 min postsulfide addition (Figures S2b and S3).

These data establish that LMW persulfides are present endogenously at less than 1% total LMW thiol under these microaerophilic culture conditions, which is ~10-fold lower than observed in *S. aureus*²⁶ under aerobic culture conditions and with thiosulfate (TS) as the sole sulfur source (Figure 3d). In addition, CoASSH is the only LMW persulfide whose ratio to total thiol increases in a statistically significant manner over time. Similar results for cellular profiling of LMW inorganic and organic species were observed when wild-type *E. faecalis* was stressed with 0.4 mM Na₂S (Figure S4).

Negative Regulation of DNA Binding by *Ef*CstR upon Reaction with CoASSH

The experiments described above make the prediction that CstR cysteine thiols would react with more electrophilic RSS to form more oxidized thiol species.¹⁶ LC-ESI-MS analysis reveals that unreacted and reduced *Ef*CstR exists mainly as a non-cross-linked monomer (12 802 Da, Figure S5a) and that anaerobic incubation with CoA, CoA disulfide, or Na₂S does

not detectably change the extent of dimer formation (Figures S5b–d). In contrast, incubation of the reduced *EfCstR* with CoASSH gives rise largely to a covalently cross-linked dimer (25 602 Da, Figure S5e), confirmed by high-resolution ESI-MS/MS in both +2 (Figure S6a) and +4 (Figure S6b) charge states. Further analysis reveals that a small amount of trisulfide-cross-linked C32- and C61-containing peptide in the +5-charge state (Figures S6c) is also present, to ~1% of the disulfide. These anaerobic derivatization experiments are consistent with initial attack of one of two Cys (C32 or C61) on CoASSH, with the release of HS[−] or CoAS[−] followed by the attack of the resolving Cys of the mixed (hydro)disulfide to generate a disulfide bond.

Fluorescent anisotropy-based DNA binding titrations were next carried out to assess the DNA binding affinities of *EfCstR* vs CoASSH-derivatized *EfCstR* to a 29-bp fluorescein-labeled DNA harboring the *cstR* operator (see Figure 1b). The binding isotherm obtained can be fit to a two-CstR tetramer binding model and reveal a significant attenuation of binding affinity for CoASSH-derivatized *EfCstR* (Figure S7a and Table 1), as previously observed for *S. aureus* CstR.^{16,43} While only CoASSH was investigated as a CstR oxidant in this way, any persulfide identified in our profiling experiments (Figure 3) could potentially react with CstR to inhibit DNA binding as found previously in *S. aureus*.^{16,43} In control experiments, *EfCstR* exposed to air for an extended period of time confirms no cysteine oxidation or formation of covalently cross-linked dimeric *EfCstR* (Figure S8).

Negative Regulation of DNA Binding by *EfCstR* upon Reaction with Angeli's Salt

Given that the *cst*-like operon in *E. faecalis* is inducible by Angeli's salt (Figure 2c), we next determined the DNA binding affinity of Angeli's salt-derivatized *EfCstR*. We find an attenuation of DNA binding affinity of *EfCstR* comparable to that of CoASSH-reacted *EfCstR* (Figure S7b and Table 1). Although the chemical modification on *EfCstR* was not investigated here, Angeli's salt is expected to induce the formation of interprotomer disulfide bridges as previously observed in *S. aureus*.⁷ These data suggest that HNO reacts directly with *EfCstR* thiols to inhibit DNA binding, thus providing a potential second mechanism beyond HNO-induced perturbation of sulfide and persulfide levels to explain the induction of the *cst*-like operon by HNO.⁷ Given similar degrees of inhibition of DNA binding by oxidation of CstR with Angeli's salt vs CoASSH, the relatively weaker induction by Angeli's salt (Figure 2) suggests that a smaller fraction of CstR thiols may be derivatized by HNO in cells.

Thiosulfate Sulfurtransferase Activities of RhdA, RhdB, and CDR-RHD

Both *rhdA* and *rhdB* are predicted to encode canonical rhodanases,⁴⁰ which are thought to transfer sulfane sulfur from a persulfide donor to an appropriate cellular acceptor(s); however, their persulfide target specificity, if any, remains undefined. Interestingly, homology models created for RhdA and RhdB reveal largely opposite electrostatic surface potentials and have been designated as such. RhdA is largely acidic, and RhdB is basic (Figure S9). These electrostatic properties may impact their as yet undefined physiological roles in maintaining cellular sulfide and RSS homeostasis of both small molecules and the proteome,¹⁸ but this was not investigated further here. As anticipated, RhdA and RhdB possess significant thiosulfate sulfurtransferase (TST) activity, with steady-state kinetic

parameters comparable to those of *S. aureus* CstA rhodanese previously characterized (Figure S9a,b).⁴²

In *S. aureus* CstA, the N-terminal rhodanese possesses significant TST activity; the same is true of the C-terminal rhodanese domain of the persulfide dioxygenase, CstB.^{37,42} As described below, the CDR-RHD encoded by *coaP* (see Figure 1a) also harbors a C-terminal rhodanese homology domain with a putative active site cysteine, C508 (Figure 4a). We find only minimal TST activity (Figure 4b); k_{cat} is some 90-fold lower than the CstA^{Rhod} and authentic rhodanases RhdA and RhdB.⁴² This suggests that the TST activity of this domain is not functionally relevant in cells, thus implicating this domain in performing some other function (*vide infra*).

The *coaP*-Encoded CDR-RHD Is a *Bona Fide* CoA Persulfide Reductase

The structures of previously characterized CDR-RHD fusions from *B. anthracis* (Figure 1a) and *Shewanella loihica*⁴⁷ reveal a homodimeric assembly state where each protomer contains an N-terminal CoA disulfide reductase (CDR) domain and a C-terminal rhodanese homology domain (RHD; Figure 4a).³⁶ As expected from these studies, fully reduced *Ef* CDR-RHD adopts a dimeric assembly state (Figure S10a).^{35,48} In contrast to a typical CDR⁴⁹ or a CDR-RHD, *Ef*CDR-RHD as purified does not show a characteristic FAD absorption (Figure S10b). As a result, stoichiometric FAD was added to the purified enzyme in order to detect any reductase activity. Although CoASSH, CysSSH, HCySSH, and GSSH are all found in cells (Figure 3), we find that only CoASSH is used by *Ef*CDR-RHD as a substrate, with a K_m of 111 (± 23) μM and k_{cat} of 4.9 (± 0.4) s^{-1} , obtained by monitoring NADH oxidation (Table 2). These steady-state parameters compare favorably with those measured previously for the polysulfide reductase from *P. furiosus*,⁴¹ yielding similar catalytic efficiencies, k_{cat}/K_m , of $4.4 (\pm 1.0) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ and $5.4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for the *Ef* CDR-RHD and *P. furiosus* enzymes, respectively.⁴¹ *Ef*CDR-RHD exhibits no detectable FAD-dependent reductase activity toward other sulfur-containing species in the CoASSH substrate mixture (see Methods), which includes CoA, CoA disulfide, or Na_2S (Figure 4c), and the observed persulfide reductase activity is specific for NADH over NADPH⁴⁷ (Table 2). A lack of activity toward CoA disulfide is consistent with the structure, which reveals a binding site for a single pantothenate arm that bridges the two domains within a pseudoprotomer (Figure 4a).

Product analysis of these CDR-RHD-catalyzed persulfide reductions reveals the expected products of a two-electron reduction of CoASSH, namely, the free thiol CoASH and HS (Figure 4e). We have therefore renamed *Ef*CDR-RHD CoA persulfide reductase “CoAPR.” Additional steady-state kinetics experiments reveal that *Ef*CDR-RHD is moderately inhibited by the product thiol CoA and exhibits some inhibition when the poor substrate CysSSH is added to these reactions (Table 2). The weak inhibition by CysSSH can be explained by the fact that cysteine, in both the thiol and persulfide forms, defines the business end of CoA and may well bind to CoAPR with some affinity. The extent to which this would occur *in vivo* is not known, but cysteine thiol and persulfide are present at lower concentrations in cells relative to those derived from CoA (Figures 3 and S2).

C42S and C508S CoAPR mutants are devoid of CoA persulfide reductase activity as they exhibit no NADH oxidation (Figure 4d), nor do they generate either of the expected products (Figure 4e). C42 is conserved in all CDRs^{48,49} and in CDR-RHDs,^{36,41,47} and forms an obligatory mixed disulfide with CoA as the redox center in the resting state of this group 3 flavoprotein disulfide reductase that is required for both disulfide and persulfide reductase activities.⁵⁰ In contrast, the role of C508 is less clear but may play a role in persulfide sulfur transfer.^{36,47} Although the two active sites of CoAPR must cooperate to generate the products of NADH oxidation (Figures 4e,f), the existing structures of CDR-RHDs (Figure 4a) provides little insight as to how this might occur, since the two active sites are far apart from one another.^{36,47} Indeed, either mechanism shown (Figure 4f) would seem to require substantial changes in the relative orientations of the CDR and RHD domains within or between protomers of the dimer, and/or a “swinging pantothenate arm” as suggested previously.^{36,47}

CoaP Complements a Sulfide-Induced Growth Phenotype in a *S. aureus* *cstB* Strain

We next investigated a potential biological role of CoAPR motivated by our characterization of CstB as a nonheme Fe^{II} persulfide dioxygenase (PDO) that catalyzes the oxidation of RSSH to thiosulfate and reduced thiols, and our finding that a *cstB* strain of *S. aureus* strain Newman exhibits a sulfide-induced growth phenotype.³⁷ This growth phenotype can be complemented by expression of the wild-type allele from an extrachromosomal plasmid (Figure 5a).³⁷ Remarkably, a wild-type-like growth phenotype is obtained in 0.2 mM NaHS stress when the *cstB* strain is complemented with the *E. faecalis* *coaP* gene encoded on the same plasmid (Figure 5b). In contrast, a plasmid encoding C42S or C508S alleles of *coaP* fails to complement the *cstB* growth phenotype, which is consistent with the essentiality of both cysteine residues in the enzymatic activity of CoAPR (Figure 4d). In addition, a near wild-type CoASSH/CoA ratio is achieved when a *cstB* strain is complemented by expression of either the wild-type *cstB* allele or the *E. faecalis* *coaP* gene encoded on a plasmid (Figure 5c).

A *cstR* *E. faecalis* Mutant Exhibits Reduced Virulence in a Murine Mouse CAUTI Model

We next investigated the effects of RSS on the pathophysiology of an *E. faecalis*-mediated catheter-associated urinary tract infection (CAUTI) model by introducing 4- to 5-mm-long pieces of silicone tubing into the bladders of C57BL/6Ncr female mice. Following catheter implantation, mice were immediately infected with $\sim 2 \times 10^7$ CFU of OG1RF wild-type (WT) or *cstR* mutant (Table S1) in phosphate-buffered saline (PBS) introduced transurethrally into the lumen of the bladder. Twenty-four hours after infection, bacteria were recovered from the bladder at a median value of 6.0×10^5 CFU for animals infected with WT OG1RF and 4.0×10^5 CFU for animals infected with the *cstR* mutant, exhibiting no statistical difference in colonization (Figure 6a). In contrast, bacteria recovered from the implants at a median value of 4.2×10^5 CFU for animals infected with WT OG1RF, and a significant decrease was observed for animals infected with *cstR* mutant, 10.7×10^4 CFU (Figure 6b). These data suggest that projected hyper-clearance of cytoplasmic RSS²⁶ in *E. faecalis* results in decreased virulence in the CAUTI model, thus implicating RSS regulation as important in the pathophysiology of this opportunistic pathogen.

DISCUSSION

Enterococci, normally commensal bacteria of the human gastrointestinal tract (GIT), have become important opportunistic pathogens. They are a leading cause of hospital-acquired urinary tract infections and are frequently associated with other severe diseases such as endocarditis and bacteremia.^{51,52} While sulfide plays a role in various physiological processes that protect the GIT,^{53–55} increased H₂S has also been implicated in several gut-associated diseases including colonic inflammation, ulcerative colitis,⁵⁶ colorectal cancer,⁵⁷ and irritable bowel syndrome.⁵⁸ A number of reports suggest that the GIT is exposed to millimolar endogenous sulfide from a variety of sources,^{59,60} thus necessitating homeostatic mechanisms that regulate cellular sulfide and associated RSS in bacteria that inhabit this niche. We propose that CstR is a *bona fide* RSS sensor^{16,43} and that *cst*-like operon-encoded proteins mediate RSS speciation in *E. faecalis*.

The importance of the expression of the *cst*-like operon genes for *E. faecalis* colonization of the GIT and pathogen virulence is not yet known, but elevated levels of sulfide can be detected in this particular niche.^{9,14,61} In addition, treatment of some bacteria with β -lactam antibiotics upregulates enzymes that biosynthesize pantothenic acid and CoA perhaps in an effort to overcome antibiotics-induced H₂S upregulation⁸ and the resulting increase in CoA persulfides (Figure 3) that occur as a result. Using a murine mouse CAUTI model, we observe a significant decrease in bacterial colonization of silicone catheter implants when mice are infected with a *cstR* mutant strain (Figure 6b). This suggests that uncontrolled clearance of RSS mediated by unregulated expression of CstR-regulated genes (Figure 2a)²⁶ reduces the virulence of this opportunistic pathogen and implies that regulation of these species in *E. faecalis* is important to its pathophysiology in this niche. It is interesting to note that mice infected with a *S. aureus cstR* mutant strain also show a virulence phenotype in the kidney, an organ of the upper urinary tract.²⁶ It is well established that urinary tract infections give rise to a significant burden of the ROS and oxidative damage in this niche,^{62–66} and the production and proper regulation of RSS may provide the bacteria a growth advantage in this microenvironment. Indeed, early studies in *S. aureus* showed that nitrite- and NO-induced dispersal of an abiotic biofilm results in upregulation of the *cst* operon,⁶⁷ consistent with our findings in the CAUTI model.

Glutathione, cysteine, CoA, and homocysteine are major constituents of the LMW thiol pool in *E. faecalis* under these growth conditions (Figure 3b), with homocysteine ~15-fold higher than in *S. aureus*.²⁶ L-Homocysteine can be formed *via* the trans-sulfuration process that converts L-cysteine, formed by cysteine synthase, to L-cystathionine *via* cystathionine γ -synthase (CGS), which is then converted to L-homocysteine *via* cystathionine- β -lyase (CBL).⁶⁸ Exogenous Na₂S clearly drives the biosynthesis of L-homocysteine (Figure 3b), likely *via* this pathway. Additionally, L-cysteine can be formed from L-homocysteine *via* the reverse transsulfuration process, which produces L-cysteine through a L-cystathionine intermediate, and L-methionine can be produced by a transmethylation reaction utilizing a methyl donor, *e.g.*, tetrahydrofolate, to methylate L-homocysteine to biosynthesize L-methionine.^{69,70} The intestinal flora are a major site of L-homocysteine production,^{69,71} which drives glutathione biosynthesis;^{72,73} exposure of gut micro-organisms to homocysteine may therefore impact sulfide and RSS homeostasis in this niche, consistent

with our profiling experiments (Figure 3). *E. faecalis* also maintains a significant pool of CoA that is strongly perturbed upon addition of exogenous sulfide to cells (Figure 3).

Our polysulfide metabolite profiling experiments reveal that CoASSH, while readily detected in ambiently grown cells, is maintained at very low endogenous levels relative to other organic persulfides, in stark contrast to what was observed in *S. aureus*.²⁶ The addition of exogenous Na₂S uniquely increases these levels by ~5–10-fold over several cell doublings (Figure 3), giving rise to a measurable growth phenotype (Figure S2c). This suggests that increases in RSS of this magnitude overruns the *cst*-like operon expression, and the organism is unable to effectively clear CoASSH and other RSS; this, in turn suggests that *E. faecalis* must protect itself from accumulating CoASSH. Consistent with this, we show here that one of the CstR-regulated genes, *coaP*, encodes a *bona fide* CoA persulfide reductase, CoAPR. Although the structure and biochemical characterization is available only for closely related enzymes,^{36,41,47} we show here that CoAPR requires both domains to reduce the hydrodisulfide of CoA, producing the free thiol CoA and H₂S in an FAD-dependent and NADH-requiring fashion (Figure 4e). In contrast to the *cst* operon of *S. aureus*, which clearly functions in sulfide detoxification, the *E. faecalis* *cst*-like operon-encoded CoAPR, which while regenerating CoA for metabolic use as in *S. aureus*, also produces H₂S, a finding that appears counter to a role in sulfide detoxification. However, the ultimate fate of this CoAPR-generated H₂S is not yet known nor is the degree to which H₂S versus CoASSH is deleterious to *E. faecalis* growth.

The finding that a sulfide-induced *cstB* growth phenotype of *S. aureus* can be complemented by a wild-type *coaP* allele, but not C42S or C508S alleles (Figure 5), strongly suggests that in *S. aureus*, the origin of the growth defect may well be an elevated level of CoASSH. In fact, a single *cst*-encoded gene (*cstB*) deletion in *S. aureus* also gives rise to a striking increase specifically of CoASSH to ~15% of the endogenous CoA pool (Figure 5c). Although we do not yet know how CoAPR is able to complement a lack of CstB, their orthogonal chemistries suggest to us that these sulfide/RSS homeostasis systems may have evolved in part to protect the integrity of the cellular CoA pool, derivatization of which will negatively impact cellular metabolism through disruption of the TCA cycle and other acyl-transfer requiring processes.⁷⁴ For example, if CoASSH is a substrate for pyruvate dehydrogenase, the product of that reaction would be the mixed disulfide between thioacetate and CoA, which would simply regenerate CoA upon cellular reduction, negatively impacting levels of short chain thioesters, including acetyl-CoA. We also show that when *E. faecalis* is stressed with Angeli's salt, a smaller but significant induction of the *cst*-like operon is observed (Figure 2b) and control experiments with NO₂⁻ and a representative NO donor suggest that HNO is the actual physiological effector of Angeli's salt, as previously established for *S. aureus*.^{7,16} In addition, since we observe a similar attenuation of DNA operator binding affinity by both Angeli's salt- and CoASSH-oxidized EfCstR, the *cst*-like operon may be capable of responding to multiple cellular stressors via similar or distinct modifications of EfCstR. Since HNO is an inducer of both operons, it will be very interesting to determine if CoAPR and CstB possess catalytic activity toward CoA sulfinamides (CoA-SONH₂) as the anticipated reaction product upon derivatization by HNO,³⁹ and as recently observed in yeast cells subjected to nitric oxide stress as the product of CoA-SNO reductase.³⁸ Experiments are underway to further evaluate these ideas.

METHODS

All experimental methods can be found in the Supporting Information (SI).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

Cst	copper-sensing operon repressor (CsoR)-like sulfurtransferase
RSS	reactive sulfur species
HNO	nitroxyl
H₂S	hydrogen sulfide
NO	nitric oxide
CoA	coenzyme A
CDR	CoA disulfide reductase
CoASSH	CoA persulfide
CysSH	cysteine
CysSSH	cysteine persulfide
GSH	glutathione
GSSH	glutathione persulfide
HCySH	homocysteine
HCySSH	homocysteine persulfide
Rhd	rhodanese
RHD	rhodanese homology domain

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Figure 1.

Identification of *cst*-like operons. (a) Genomic organization of the *E. faecalis* strain OG1RF *cst*-like operon region (top) compared to the previously characterized genomic region of *S. aureus* (middle)¹⁶ and an analogous region of *B. anthracis* str. Sterne (bottom) derived from a Synteny analysis using either *cstR* or *Ef coaP* genes as query.⁷⁵ *B. anthracis* str. Ames (not shown) is organized as in the Sterne strain. Genes are shaded according to function: red, sulfurtransferase repressor CstR;⁴³ yellow, sulfurtransferase or sulfur trafficking function analogous to that found in the multidomain sulfurtransferase *S. aureus* CstA (Rhd-TusA-TusD);⁴² green, nonheme Fe^{II} persulfide dioxygenase–rhodanese fusion CstB;³⁷ light green, persulfide reductase–rhodanese fusion;³⁶ blue, candidate sulfite efflux transporter TauE. In *E. faecalis*, the *cst*-like operon is composed of a *cstR* (OG1RF_RS11640, 86 residues), *rhdb* (OG1RF_RS11635, 104 residues), a candidate CoA persulfide reductase *coaP* (OG1RF_RS11630, 549 residues), and *rhda* (OG1RF_RS11625, 99 aa). The structurally characterized *B. anthracis* CDR-RHD is encoded by locus tag BAS0774, which is analogous to BA0736 shown here.³⁶ (b) Nucleotide sequence upstream of the *Ef rhdB* ORF to show the characteristic CstR operator.⁴³ The consensus 16-bp operator site for *EfCstR*⁴³ is highlighted in red bold with the 29-bp *EfCstR* duplex DNA used for the DNA binding assays underlined.

Figure 2.

E. faecalis *cst*-like operon induced by both sulfide and Angeli's salt *in vivo*. Quantitative RT-PCR was performed on (a) the *cstR* mutant and wild-type *E. faecalis* strain OG1RF with the addition of (b) 0.2 mM Na_2S and (c) 0.3 mM Angeli's salt for early mid log cultures. The fold changes of induction for *Ef* *cstR* (orange), *rhdB* (red), *coaP* (green), and *rhdA* (blue) were normalized relative to the level of *gyrase*. Values represent relative transcript levels to untreated wild-type cells and are shown as mean $\pm$ SD from replicate cultures with statistical significance established using a paired *t* test relative to unstressed wild-type strain under the same conditions (****p*<0.001, ***p*<0.01, **p*<0.05). Primers for the analysis are listed in Table S1.

Figure 3.

LMW inorganic species and organic thiol and persulfide concentrations for wild-type *E. faecalis* strain OG1RF following induction of the *cst*-like operon at $t = 0$ by 0.2 mM Na₂S. (a) Level changes of inorganic species H₂S (orange), HSSH (gray), and HSSSSH (pink). (b) Level changes of reduced glutathione (GSH, blue), cysteine (CysSH, red), CoA (white), and homocysteine (HCySH, yellow). (c) Level changes of glutathione (GSSH, blue), cysteine (CysSSH, red), CoA (CoASSH, white), and homocysteine (HCySSH, yellow) persulfides. (d) Ratio of persulfide to total cellular LMW thiol of glutathione (blue), cysteine (red), CoA (white), and homocysteine (yellow). Values represent mean $\pm$ SD of the mean derived from results of triplicate experiments with statistical significance established using a paired t test relative to wild-type strain under the same conditions (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; ns, no significant difference). For endogenous levels ($t = 0$), values represent means $\pm$ SD from $n = 12$ measurements, and values outside one standard deviation of the mean were excluded in final mean and standard deviation calculations. Additional time points for these conditions and cellular concentrations of wild-type *E. faecalis* strain OG1RF stressed with 0.4 mM Na₂S are found in Figures S2 and S4.

Figure 4.

Structural model and function of *Ef*CDR-RHD. (a) *B. anthracis* CDR-RHD adopts a homodimeric assembly state, shown in ribbon representation (PDB: 3ICT).³⁶ The N-terminal CDR domains (residues 1–450) from two protomers are shaded in *purple* and *green*, respectively, with the RHD (residues 451–554) colored in *cyan*. Each CDR-RHD protomer binds to one CoA and one FAD molecule. Active site cysteine residues from CDR (C44; C42 in *Ef*CDR-RHD) and RHD (C514; C508 in *Ef*CDR-RHD) are highlighted as spheres. (b) The initial thiosulfate turnover rate as a function of thiosulfate concentration catalyzed by *Ef*CDR-RHD, *S. aureus* CstA^{Rhod}, and CstA^{RhodTusA}, with the continuous fit to the Michaelis–Menten equation shows *Ef*CDR-RHD has minimal TST activity. *Ef*CDR-RHD K_m determined as 1.0 ± 0.6 mM and V_{max} determined as 0.08 ± 0.1 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$, and k_{cat} is some 90-fold lower than CstA^{Rhod} and CstA^{RhodTusA}.⁴² (c) The initial NADH turnover rate plotted as a function of the concentration of CoASSH, CoA, CoA disulfide, and Na₂S, with the continuous line a fit to the Michaelis–Menten eq (Table 2) and demonstrating *Ef*CDR-RHD has authentic NADH and FAD-dependent persulfide reductase activity only toward CoASSH. (d) The initial NADH turnover rate plotted as a function of the concentration of wild-type (WT), C42S, and C508S CDR-RHDs, with the continuous lines fit to the Michaelis–Menten eq (Table 2) shows no detectable persulfide reductase activity for either cysteine mutant compared to wild-type. (e) Product analysis for persulfide activity of WT, C42S, and C508S CDR-RHDs where only WT shows an increase in both CoA and H₂S products compared to C42S and C508S CDR-RHDs and control experiments without the addition of enzyme. Values represent mean $\pm$ SD derived from replicate experiments with statistical significance established using a paired *t*-test relative to control

under the same conditions ($**p = 0.01$, $*p = 0.05$). (f) Possible models for *Ef*CDR-RHD as a coenzyme A persulfide reductase, CoAPR acting upon a single CoASSH molecule. *Top route*: C42 attacks the CoASSH to form a C42-S-S-CoA mixed disulfide with the release of HS⁻, and C508 then reduces the mixed disulfide with the release of CoA and formation of C42-S-S-C508 disulfide. *Bottom route*: C508 attacks the CoASSH to form an enzyme-bound persulfide with CoA, and then C42 attacks the C508 persulfide with the release of HS⁻, forming C42-S-S-C508 disulfide. In both cases, FADH₂ eventually reduces the disulfide bond between C42 and C508, which is rereduced by NADH, and the enzyme turns over. An alternative model that incorporates features of the bottom route that invokes a CoA thiol as a cofactor has also been proposed.⁴⁷

Figure 5.

Complementation of sulfide-induced growth phenotype and increased CoASSH levels in a *cstB* *S. aureus* Newman strain by *E. faecalis* CoAPR. Representative growth curves for wild-type (WT), *cstB*, and plasmid-complemented *cstB* allelic *S. aureus* Newman strains on HHWm media in the presence of 0 mM and 0.2 mM NaHS added at $t = 0$ h. (a) WT (black squares), *cstB* (blue squares), and *cstB::pCstB* (red squares) strains in the presence of 0 mM NaHS; WT (black circles), *cstB* (blue circles), and *cstB::pCstB* (red circles) strains in the presence of 0.2 mM NaHS. (b) *cstB::pCoAPR* (violet squares), *cstB::pCoAPR^{C42S}* (green squares), and *cstB::pCoAPR^{C508S}* (pink squares) strains in the presence of 0 mM NaHS; *cstB::pCoAPR* (violet circles), *cstB::pCoAPR^{C42S}* (green circles), and *cstB::pCoAPR^{C508S}* (pink circles) strains in the presence of 0.2 mM NaHS. (c) Ratio of CoASSH to total CoA thiol for WT (black), *cstB* (gray), *cstB::pCstB* (red), and *cstB::pCoAPR* (blue). Values represent mean $\pm$ SD of the mean derived from replicate experiments with statistical significance established using a paired *t*-test relative to wild-type strain under the same conditions (** $p < 0.01$, * $p < 0.05$).

Figure 6.

A *cstR* mutant strain deficient in colonization of silicone implants in a murine model of CAUTI. Graphs represent bacterial titers in log scale recovered from the (a) bladders and (b) silicone implants from female C57BL/6Ncr mice infected with OG1RF wild-type or *cstR* mutant for 24 h. Horizontal dashed lines represent the limits of detection for viable bacteria. Each symbol represents the value for an individual mouse, and each experiment was done three times with four or five mice for each strain. The horizontal bars indicate the median value for each group of mice. Values that are significantly different by the Mann–Whitney U test are indicated as follows: * $p < 0.05$; ns, difference not statistically significant.

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Equilibrium Binding Parameters for Reduced *Ef*CstR and *Ef*CstRs Derivatized with CoASSH and Angeli's Salt^a

Table 1

reactant	r_0	r_{complex}	$K_1^b (\times 10^7 \text{ M}^{-1})$	$K_2^b (\times 10^7 \text{ M}^{-1})$	$K_{\text{tet}}^b (\times 10^7 \text{ M}^{-1})$
	0.073	0.121	1.7 ± 0.9	42 ± 7	8.4 ± 2.3
CoASSH	0.079	0.126^c	2.8 ± 0.4	0.2 ± 0.1	0.7 ± 0.1
Angeli's salt	0.077	0.124^c	0.7 ± 0.2	0.4 ± 0.1	0.5 ± 0.1

^aConditions: 10 nM fluorescein–DNA duplex, 2–240 nM *Ef*CstR (protomer), 25 mM HEPES, 200 mM NaCl, at pH 7.0, 2 mM EDTA, at 25 °C (2 mM TCEP added for the reduced *Ef*CstR).

^bDetermined from a model that assumes two tetramers bind to each operator DNA with stepwise association constants of K_1 and K_2 , and K_{tet} is the average macroscopic tetramer association constant ($K_{\text{tet}} = \text{sqrt}(K_1 \cdot K_2)$).¹⁶

^cNormalized to a change in anisotropy for unreacted *Ef*CstR.⁴³

Persulfide Reductase Activity of CoAPR towards LMW Persulfide Substrates in the Presence of FAD and Either NADH^a or NADPH^b and in the Presence of Excess Product or Other Persulfides

Table 2

LMW persulfide substrate	reaction additives	K_m (μ M)	V_{max} (μ mol min ⁻¹ mg ⁻¹)	k_{cat} (s ⁻¹)	k_{cat}/K_m ($\times 10^4$ M ⁻¹ s ⁻¹)	k_{cat} ratio ^f
CoASSH ^a	none	111 $\pm$ 23	4.9 $\pm$ 0.4	4.9 $\pm$ 0.4	4.4 ($\pm$ 1.0)	1.0 $\pm$ 0.12
CoASSH ^b	NADPH	N.D. ^c	0.37 $\pm$ 0.12	0.31 $\pm$ 0.07	N.D.	0.06 $\pm$ 0.24
CoASSH ^a	200 μ M CoASH	111 ^e	4.0 $\pm$ 0.3	4.0 $\pm$ 0.3	N.D.	0.82 $\pm$ 0.11
CoASSH ^a	1 mM CoASH	111 ^e	2.2 $\pm$ 0.2	2.2 $\pm$ 0.2	N.D.	0.45 $\pm$ 0.12
CoASSH ^a	200 μ M CysSSH	111 ^e	5.3 $\pm$ 0.4	5.2 $\pm$ 0.4	N.D.	1.1 $\pm$ 0.11
CoASSH ^a	1 mM CysSSH	111 ^e	3.4 $\pm$ 0.4	3.4 $\pm$ 0.4	N.D.	0.69 $\pm$ 0.14
CysSSH ^{a,d}	none	270 $\pm$ 568	0.11 $\pm$ 0.11	0.11 $\pm$ 0.11	N.D.	0.022 $\pm$ 1.0
GSSH ^{a,d}	none	150 $\pm$ 293	0.08 $\pm$ 0.06	0.08 $\pm$ 0.06	N.D.	0.016 $\pm$ 0.75

^a Conditions: 100 nM CoAPR (protomer), 100 nM FAD, 100 μ M NADH, 25 mM Tris-HCl, 200 mM NaCl, pH 8.0, 25 °C.

^b Conditions: 100 nM CoAPR (protomer), 100 nM FAD, 100 μ M NADPH, 25 mM Tris-HCl, 200 mM NaCl, pH 8.0, 25 °C.

^c N.D., not determined.

^d Very low activity, making it difficult to measure K_m and V_{max} accurately.

^e K_m fixed to 111 μ M during data fitting.

^f Relative k_{cat} determined relative to k_{cat} for CoASSH substrate and NADH cofactor, first row of table.