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H_2S, a Bacterial Defense Mechanism against the Host Immune Response

Tracy Toliver-Kinsky,[a,b] Weihua Cui,[a] Gabor Törö,[a] Seung-Jin Lee,[a] Konstantin Shatalin,[c] Evgeny Nudler,[c] Csaba Szabo[a,b,d]

[a]Department of Anesthesiology, University of Texas Medical Branch, Galveston, Texas, USA
[b]Shriners Hospitals for Children, Galveston, Texas, USA
[c]Department of Biochemistry and Molecular Pharmacology, Howard Hughes Medical Institute, New York University School of Medicine, New York, New York, USA
[d]Chair of Pharmacology, Department of Medicine, University of Fribourg, Fribourg, Switzerland

Address correspondence to Tracy Toliver-Kinsky, ttoliver@utmb.edu.

ABSTRACT The biological mediator hydrogen sulfide (H_2S) is produced by bacteria and has been shown to be cytoprotective against oxidative stress and to increase the sensitivity of various bacteria to a range of antibiotic drugs. Here we evaluated whether bacterial H_2S provides resistance against the immune response, using two bacterial species that are common sources of nosocomial infections, *Escherichia coli* and *Staphylococcus aureus*. Elevations in H_2S levels increased the resistance of both species to immune-mediated killing. Clearances of infections with wild-type and genetically H_2S-deficient *E. coli* and *S. aureus* were compared *in vitro* and in mouse models of abdominal sepsis and burn wound infection. Also, inhibitors of H_2S-producing enzymes were used to assess bacterial killing by leukocytes. We found that inhibition of bacterial H_2S production can increase the susceptibility of both bacterial species to rapid killing by immune cells and can improve bacterial clearance after severe burn, an injury that increases susceptibility to opportunistic infections. These findings support the role of H_2S as a bacterial defense mechanism against the host response and implicate bacterial H_2S inhibition as a potential therapeutic intervention in the prevention or treatment of infections.

KEYWORDS antibiotic resistance, burn, hydrogen sulfide, opportunistic infections

Hydrogen sulfide (H_2S) is a gaseous biological mediator that regulates important functions in the nervous, cardiovascular, immune, and gastrointestinal systems. The importance of H_2S as an endogenous mediator in mammalian systems is highlighted by discoveries that disruptions in H_2S homeostasis are associated with a wide range of disease states, including cardiovascular diseases, diabetes, burn injury, ischemia-reperfusion, and cancer (1). In mammalian cells, H_2S is produced by three enzymes: cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST) (1, 2).

Bacteria can also produce H_2S via orthologous enzymes (3). While bacterial H_2S production was long perceived primarily as a metabolic by-product, recent studies have implicated H_2S as an important signaling molecule in bacteria. H_2S can protect bacteria from antibiotic-induced damage, at least in part by sequestering free iron to prevent the Fenton reaction that generates toxic hydroxyl radicals (3, 4). Additionally, H_2S can regulate intracellular cysteine, which can be toxic at high levels (4). Recently, it was demonstrated that H_2S, and downstream reactive sulfur species, can regulate the expression of some bacterial virulence genes through S-sulfhydration of the proteome (5). Given the importance of reactive oxygen species (ROS) in antimicrobial immune responses, especially innate responses (6), we have now evaluated whether bacterial H_2S protects bacteria from the host immune response by manipulating H_2S levels in bacteria without modulation of host H_2S. The findings in this report support the role of

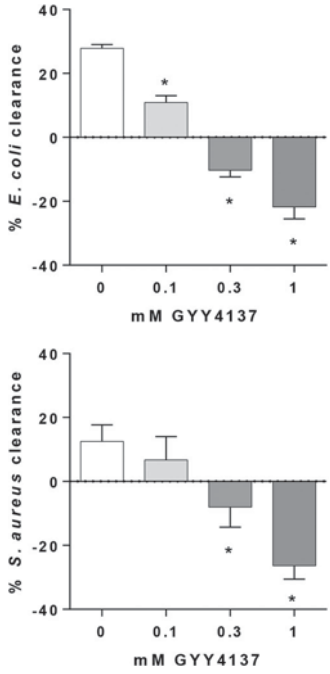

FIG 1 *E. coli* and *S. aureus* were cultured with increasing concentrations of up to 1 mM GYY4137 prior to inoculation of mouse leukocyte cultures. Graphs show percentages of inoculum CFU killed after coculture with leukocytes. *, significantly different from 0 mM ($n = 4$ to 5 replicates per group).

H_2S as a bacterial defense mechanism and implicate bacterial H_2S inhibition as a potential therapeutic intervention for the treatment or prevention of infections.

RESULTS

Because manipulation of host H_2S production could have potential effects on the immune response to infection, experiments were designed to specifically manipulate bacterial, but not host, H_2S levels. To determine if pharmacological donation of H_2S can increase the resistance of bacteria to the immune response, *Escherichia coli* and *Staphylococcus aureus* were cultured in the presence or absence of a slow-release H_2S donor, GYY4137 (7). GYY4137 is an organic small molecule that slowly decomposes to release low levels of H_2S over a prolonged period of time. Its use as a low-level H_2S generator in biological systems, both mammalian and nonmammalian, has been well characterized (8, 9). To prevent any direct effects of GYY4137 on host cells, bacteria were preincubated with the H_2S donor, which was removed prior to inoculation of the mouse leukocyte cultures. H_2S donation to *E. coli* concentration-dependently reduced leukocyte-mediated clearance of *E. coli in vitro* ($P < 0.05$) (Fig. 1). Bacterial clearance was completely prevented at the highest concentrations of GYY4137 tested (0.3 and 1 mM), allowing bacterial growth in the presence of leukocytes, suggesting that H_2S can protect *E. coli* from immune-mediated killing. Similarly, at concentrations of 0.3 and 1 mM, GYY4137 significantly reduced and prevented rapid killing of *S. aureus in vitro* ($P < 0.05$) (Fig. 1). This was not due to effects of GYY4137 on bacterial proliferation rates, as bacterial counts were similar in the corresponding control cultures lacking leukocytes (not shown).

Since pharmacological donation of H_2S increased the resistance of both *E. coli* and *S. aureus* to killing by leukocytes, we attempted to pharmacologically inhibit H_2S production in these two bacterial species. *E. coli* lacks homologues for the mammalian H_2S-producing enzymes CBS and CSE but expresses the 3-MST homologue that is encoded by the *sseA* gene (3). Because there are currently no inhibitors available that are specific for bacterial 3MST, an inhibitor of mouse 3-MST (10, 11) was preincubated with *E. coli*, and removed, prior to inoculation of leukocytes. *E. coli* production of H_2S,

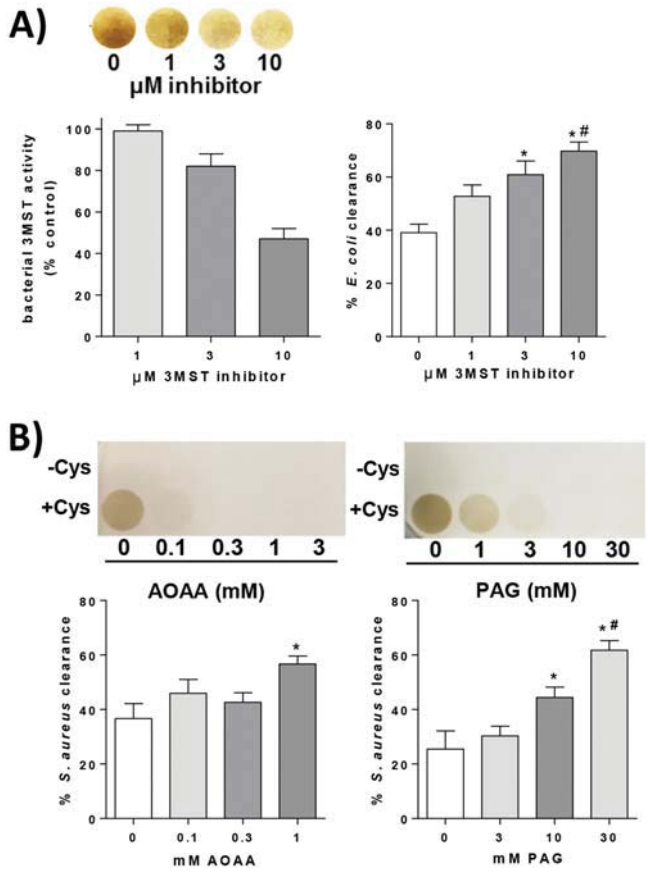

FIG 2 (A) Image showing brown lead sulfide staining produced by reaction of lead acetate with H_2S produced by *E. coli* cultured with or without the 3-MST inhibitor. The graph on the left shows the activity of bacterial 3MST in the presence of increasing concentrations of the 3-MST inhibitor, expressed as a percentage of 3MST activity in the control (no inhibitor) ($n = 3$ replicates/group). The graph on the right shows *in vitro* elimination of *E. coli* by leukocytes after 45 min. WT *E. coli* bacteria were cultured in the presence of the 3-MST inhibitor prior to inoculation of leukocytes. *, significantly different from 0 μM; #, significantly different from 1 μM ($n = 5$/group). (B) Brown lead sulfide stain produced by reaction of lead acetate with H_2S from *S. aureus* cultured with or without increasing concentrations of AOAA or PAG, in the presence (+Cys) or absence (−Cys) of 200 μM cysteine supplementation. Graphs show *in vitro* elimination of *S. aureus* by leukocytes after 60 min. WT *S. aureus* bacteria were cultured with inhibitors prior to inoculation of leukocytes. *, significantly different from 0 mM; #, significantly different from 3 mM ($n = 5$/group).

detected by reaction with lead acetate, was decreased but not completely inhibited in the presence of increasing concentrations of the 3-MST inhibitor. The ability of this inhibitor to inhibit bacterial 3MST was confirmed, as the activity of purified bacterial 3MST was decreased but not completely inhibited in the presence of increasing concentrations of the 3-MST inhibitor (Fig. 2A). *In vitro* clearance of *E. coli* was significantly increased in the presence of the 3-MST inhibitor, in a dose-dependent manner ($P < 0.05$) (Fig. 2A).

S. aureus lacks 3MST but expresses CBS and CSE homologues (3). It was observed that the level of H_2S production by *S. aureus* was markedly lower than that of *E. coli* and nearly undetectable by reaction with lead. However, supplementation of cultures with 200 μM cysteine, a substrate for both CBS and CSE, increased H_2S production to detectable levels (Fig. 2B). Because bacterium-specific CBS and CSE inhibitors are not available, commonly used inhibitors of the mammalian homologues were used in an attempt to decrease H_2S production by *S. aureus*. Amino-oxyacetic acid (AOAA) is an inhibitor of human CBS that also has some inhibitory activity against human CSE. Propargylglycine (PAG) inhibits human CSE but not CBS (12). These inhibitors were previously demonstrated to reduce H_2S production by *S. aureus* (3). *S. aureus* bacteria

were preincubated with either AOAA or PAG, which was subsequently removed prior to inoculation of leukocytes. As shown in Fig. 2B, AOAA effectively decreased H_2S production in *S. aureus* cultures. AOAA (at a concentration of 1 mM) increased the clearance of *S. aureus* by leukocytes ($P < 0.05$) (Fig. 2B). Similarly, H_2S production by *S. aureus* was inhibited by PAG in a dose-dependent manner, as was *in vitro* clearance of *S. aureus* by leukocytes ($P < 0.05$) (Fig. 2B).

Because the 3-MST, CBS, and CSE inhibitors used here would inhibit the activity of the respective enzymes in host (mouse) cells, which could potentially affect the host response to infection, we were unable to include the inhibitors during the *in vitro* bacterial killing assays; therefore, rapid bacterial clearance was assessed immediately after the removal of the inhibitors from bacteria and within a short time frame, during which bacterial proliferation was negligible. Additionally, we were unable to treat infected mice to determine the therapeutic potential of bacterial 3MST, CBS, or CSE inhibition for clearance of *E. coli* or *S. aureus* infections *in vivo*, as continued treatment with inhibitors would be required while bacteria replicate and disseminate *in vivo*, making it difficult to distinguish effects caused by bacterial versus host H_2S inhibition. Therefore, bacteria that are genetically deficient in specific H_2S-synthesizing enzymes were utilized for further studies.

To establish a role of bacterial 3MST in *E. coli* defense against the immune response, wild-type (WT) *E. coli* and *sseA*-deficient *E. coli* strains were utilized. As shown in Fig. 3A, H_2S production by 3MST-deficient *E. coli* (Δ*sseA*) is negligible compared to that by WT *E. coli*. Additionally, the level of rapid clearance of *E. coli* by leukocytes *in vitro* was significantly higher in cultures inoculated with the Δ*sseA* strain (45% bacterial clearance) than in those inoculated with the WT (12% clearance; $P < 0.05$) (Fig. 3A). Levels of bacterial proliferation were negligible during the bacterial clearance assay and were similar between WT *E. coli* and the Δ*sseA* strain. The susceptibility of *sseA*-deficient *E. coli* bacteria was reversed when they were preincubated in the presence of the H_2S donor ($P < 0.05$) (Fig. 3B), and bacterial clearance was completely prevented at higher GYY4137 concentrations (0.3 and 1 mM).

Significant differences in the clearance of opsonized bacteria by total leukocytes were detected within an hour, suggesting H_2S-mediated protection from early innate immune responses. To further define a role of 3MST in *E. coli* defense against phagocytic killing, the macrophage-like RAW 264.7 cell line was used. The 3MST-deficient *E. coli* bacteria were killed more rapidly than WT *E. coli* bacteria. Specifically, in cultures inoculated with WT *E. coli*, 38% of the bacteria were killed by 1 h, and 64% were killed by 2 h (Fig. 3C), whereas 68% of Δ*sseA* strain bacteria were killed by 1 h, and 84% were killed by 2 h. To compare resistances of the two strains to intracellular killing, intracellular viability was measured by a gentamicin protection assay. At the start of the assay (time zero), which was 1.5 h following inoculation of macrophage cultures with *E. coli*, there were significantly more viable intracellular wild-type *E. coli* bacteria than Δ*sseA* strain bacteria, and the numbers of viable intracellular WT *E. coli* bacteria remained steady throughout the assay, whereas numbers of viable intracellular *E. coli* Δ*sseA* bacteria consistently decreased and were significantly lower at all time points ($P < 0.05$) (Fig. 3C).

To determine if deficiency in H_2S-producing enzymes similarly increases susceptibility of *S. aureus* to immune responses, clearance of wild-type and *cbs*- and *cse*-deficient *S. aureus in vitro* was measured when levels of proliferation were negligible and similar between the two strains (Fig. 4A). As observed previously, the level of *S. aureus* production of H_2S was low in the absence of cysteine supplementation. Figure 4A shows a very faint precipitate with WT *S. aureus* that was not observed with the Δ*cbs* Δ*cse* strain. Supplementation of cultures with cysteine increased H_2S production to detectable levels, with substantially lower levels being produced by *cbs*- and *cse*-deficient *S. aureus*. The rate of bacterial clearance was significantly higher in cultures inoculated with the Δ*cbs* Δ*cse* strain (71%) than in cultures inoculated with WT *S. aureus* (23% elimination; $P < 0.05$) (Fig. 4A). The susceptibility of Δ*cbs* Δ*cse* strain bacteria to

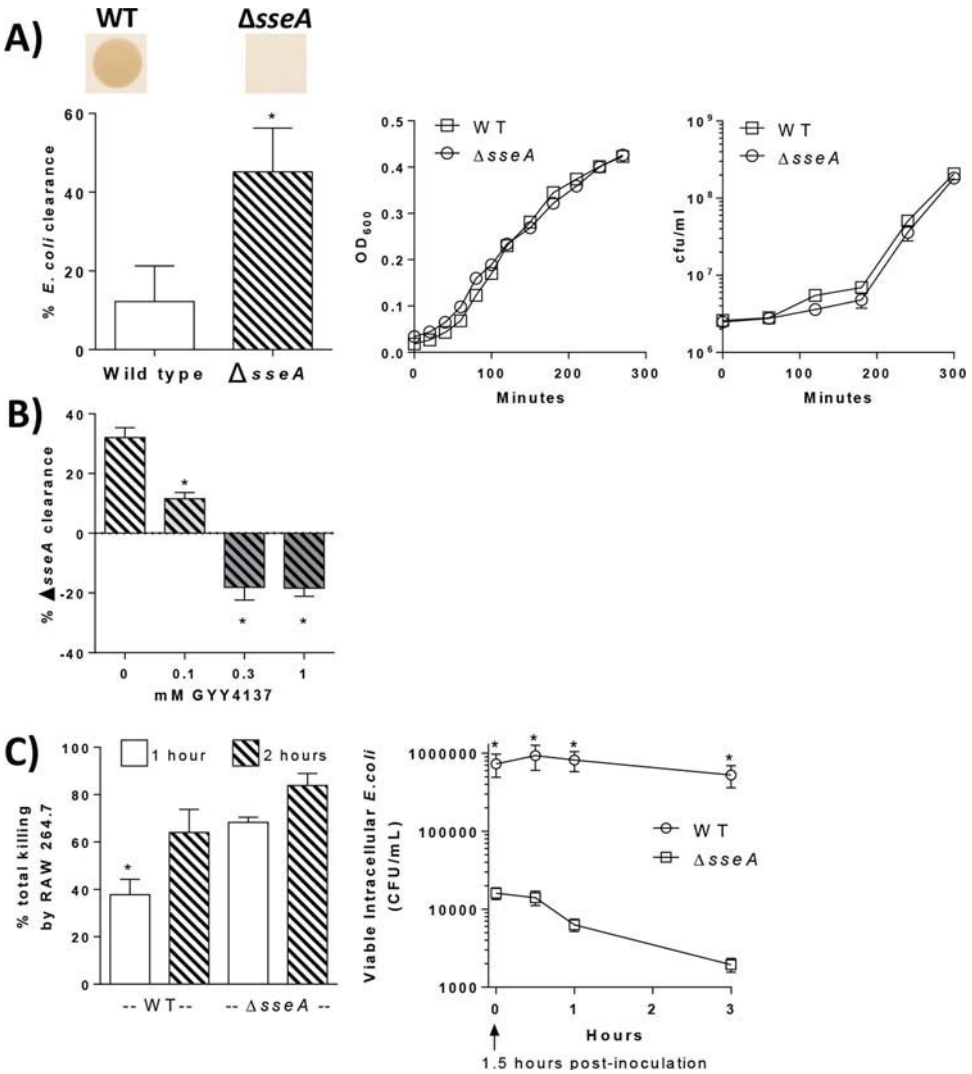

FIG 3 (A) Images showing brown lead sulfide staining produced by reaction of lead acetate with H_2S produced by bacterial cultures. WT, wild-type *E. coli*; Δ*sseA*, *sseA*-deficient *E. coli*. (Left) Bacterial elimination by leukocytes after 45 min *in vitro*. *, significantly different from the WT ($n = 6$/group). (Right) Densities of *E. coli* WT and Δ*sseA* bacteria in liquid cultures over 5 h, measured by the OD_{600}, and viability in cell culture medium, measured as CFU per milliliter ($n = 3$/group). (B) *E. coli* Δ*sseA* bacteria were cultured in the presence of GYY4137 prior to inoculation of mouse leukocytes. The graph shows bacterial elimination after 45 min of coculture. *, significantly different from 0 mM ($n = 4$ per group). (C, left) Total killing of *E. coli* bacteria by RAW 264.7 macrophages after 1 and 2 h *in vitro*. *, significantly different from all other groups ($n = 7$/group). (Right) Levels (CFU per milliliter) of viable intracellular *E. coli* bacteria recovered from RAW 264.7 cells. *, significantly different from the Δ*sseA* group at the corresponding time point ($n = 6$/group and time point).

leukocytes was reversed in the presence of increasing concentrations of the H_2S donor GYY4137 ($P < 0.05$) (Fig. 4B).

To determine if H_2S deficiency affects bacterial clearance *in vivo*, mice were infected intraperitoneally (i.p.) with WT or H_2S-deficient bacteria. Bacterial burden and systemic levels of interleukin-6 (IL-6), a marker of systemic inflammation and an indicator of poor outcomes during sepsis in mice and humans (13, 14), were measured 16 h later. When mice were inoculated i.p. with equal numbers of WT or 3MST-deficient *E. coli* bacteria, all mice infected with WT *E. coli* developed bacteremia, with a mean bacterial burden of 1.5×10^3 CFU/ml in the blood, whereas only 1 of 5 mice inoculated with the Δ*sseA* strain had a positive blood culture, which was negligible (200 CFU/ml). The bacterial burden in the spleen was similarly and significantly lower in Δ*sseA* strain-infected mice (3.4×10^5 CFU/g in the Δ*sseA* strain group versus 2.5×10^7 CFU/g in the WT *E. coli*

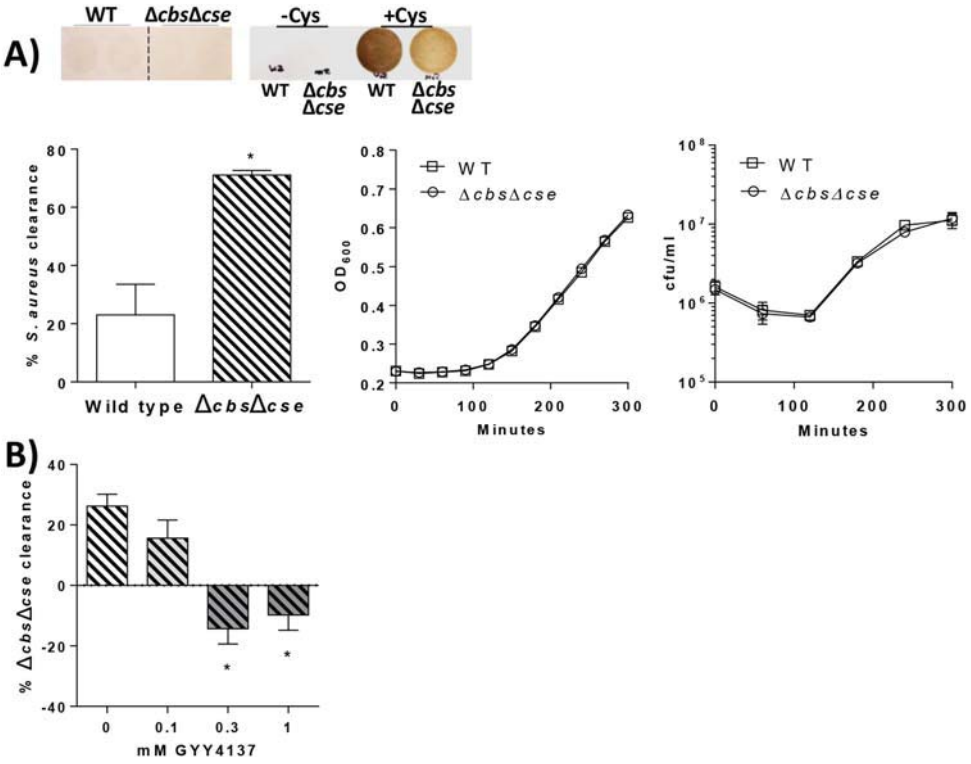

FIG 4 (A) Images showing brown lead sulfide staining produced by reaction of lead acetate with H_2S produced by bacteria grown with or without cysteine (Cys) (200 μM) supplementation. WT, wild-type *S. aureus*; Δ*cbs* Δ*cse*, *S. aureus* lacking the *cbs* and *cse* genes. The graph on the left shows bacterial clearance by leukocytes after 60 min *in vitro*. *, significantly different from the WT ($n = 6$/group). The graphs on the right show densities of *S. aureus* WT and Δ*cbs* Δ*cse* bacteria in liquid cultures over 5 h, measured by the OD_{600}, and viability in cell culture medium, measured as CFU per milliliter ($n = 3$/group). (B) *S. aureus* Δ*cbs* Δ*cse* bacteria were cultured in the presence of GYY4137 prior to inoculation of mouse leukocytes. The graph shows the percentage of the inoculum cleared after coculture with leukocytes. *, significantly different from 0 and 0.1 mM ($n = 5$ per group).

group; $P < 0.05$) (Fig. 5A). Additionally, systemic levels of IL-6 were lower in Δ*sseA* strain-infected mice (372 ± 17.9 pg/ml) than in those infected with WT *E. coli* (1,480 ± 175 pg/ml; $P < 0.05$). This was not caused by differences in basic growth rates between the two strains, as levels of proliferation over 16 h (time frame of the *in vivo* infection) were similar between *E. coli* WT and Δ*sseA* bacteria (Fig. 5A). Similarly, when mice were given an i.p. inoculation with equal numbers of *S. aureus* WT and Δ*cbs* Δ*cse* bacteria, mean bacterial counts in the spleen 16 h later were significantly lower in Δ*cbs* Δ*cse* strain-infected mice (4.4×10^6 CFU/g) than in mice infected with WT *S. aureus* (4.3×10^7 CFU/g; $P < 0.05$) (Fig. 5B). Additionally, there was a significant reduction in the level of systemic inflammation marker IL-6 in Δ*cbs* Δ*cse* strain-infected mice compared to WT-infected mice (2,950 ± 1,222 pg/ml for the wild type; 170.0 ± 6.401 pg/ml for the Δ*cbs* Δ*cse* strain; $P < 0.05$) (Fig. 5B). Bacterial growth rates were nearly identical between the two strains within the first 8 h, after which time growth of the Δ*cbs* Δ*cse* strain was slightly slower than that of the WT strain (Fig. 5B).

To determine if bacterial H_2S deficiency has therapeutic potential under conditions in which immune responses to infection are deficient, bacterial clearance was measured in mouse models of burn injury-associated infections. Burn patients are susceptible to life-threatening infections, due largely to burn-induced alterations in immune function that decrease patient defense against opportunistic microorganisms, and similar immunological perturbations occur in mice after burns. First, burn-injured mice were infected i.p. with *E. coli* as a model of burn-associated bacterial peritonitis. Peritonitis frequently develops into abdominal sepsis, and *E. coli* is a common contributor (15). As

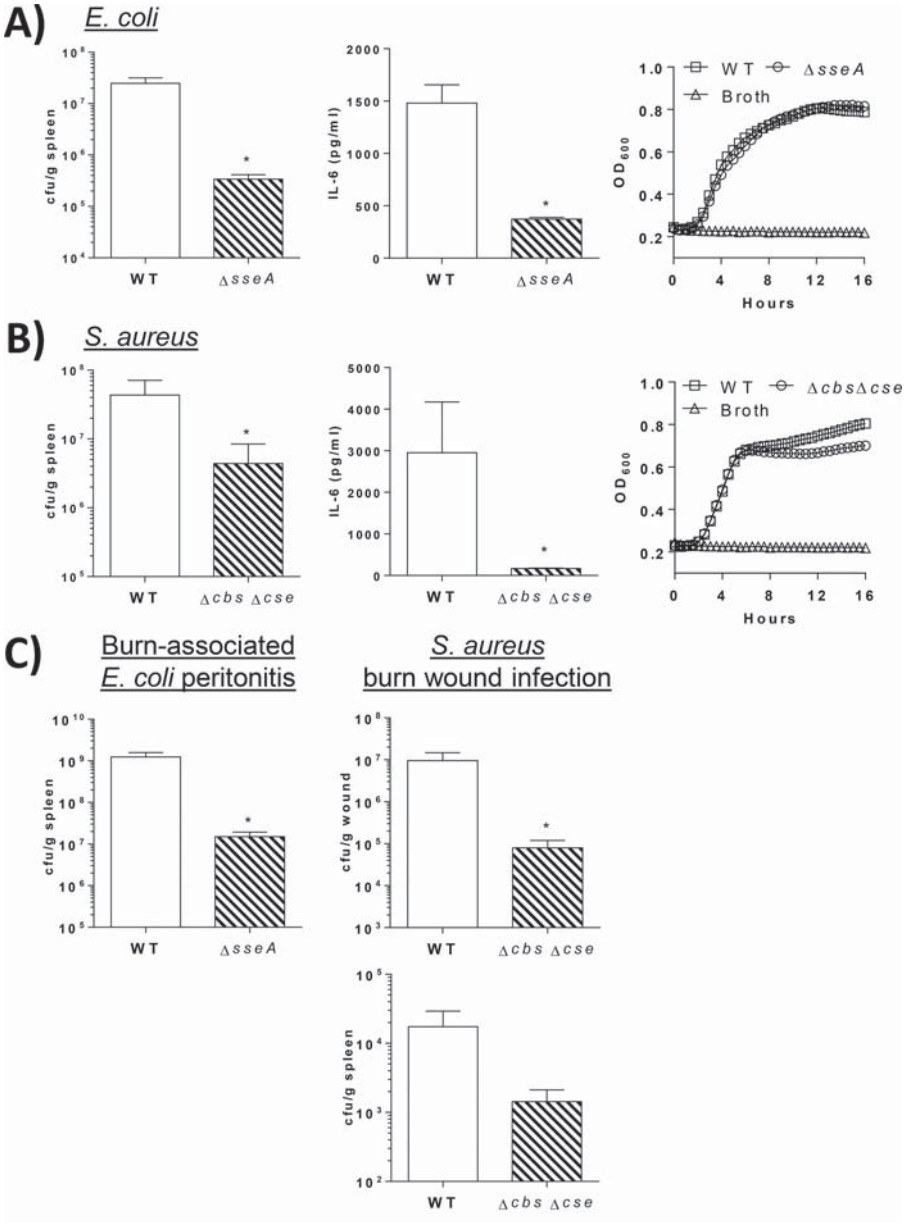

FIG 5 (A, left) Bar graphs showing bacterial counts in spleen and IL-6 levels in plasma 16 h after i.p. injection of mice with 1×10^8 CFU *E. coli*. *, significantly different from the WT ($n = 5$ mice/group). (Right) Densities of *E. coli* WT and Δ*sseA* bacteria in liquid cultures, measured by the OD_{600} over 16 h. (B, left) Bar graphs showing bacterial counts in spleens and plasma IL-6 levels in mice 16 h after i.p. injection of 5×10^8 CFU *S. aureus*. *, significantly different from the WT ($n = 5$/group). (Right) Densities of *S. aureus* WT and Δ*cbs* Δ*cse* bacteria in liquid cultures, measured by the OD_{600} over 16 h. (C, left) Graph showing bacterial counts in spleens of burned mice 16 h after i.p. injection of mice with 1×10^8 CFU *E. coli*. *, significantly different from the WT ($n = 5$ mice/group). (Right) Graphs showing bacterial counts in burn wounds and spleens of mice 24 h after inoculation of wounds with 1×10^6 CFU *S. aureus*. *, significantly different from the WT ($n = 3$ to 4/group).

shown in Fig. 5C, the bacterial burden was significantly lower in burned mice infected with the Δ*sseA* strain (1.5×10^7 CFU/g) than in mice infected with WT *E. coli* (1.2×10^9 CFU/g) ($P < 0.05$). Next, the effects of bacterial H_2S deficiency on clearance of *S. aureus* burn wound infection were examined. Wound infections are a common contributor to sepsis in burn patients, and methicillin-resistant *S. aureus* (MRSA) is a common early colonizer of burn wounds (16, 17). Burn wounds were inoculated with *S. aureus* WT or Δ*cbs* Δ*cse* bacteria, and bacterial growth within the wound and dissemination

were measured. At 24 h postinoculation, there were significantly fewer bacteria in the wounds of mice inoculated with the Δ*cbs* Δ*cse* strain (7.9×10^4 CFU/g) than in mice inoculated with WT *S. aureus* (9.6×10^6 CFU/g) ($P < 0.05$) (Fig. 5C). Similarly, the bacterial burden in the spleen was lower in Δ*cbs* Δ*cse* strain-infected mice than in those infected with WT *S. aureus* (1.4×10^3 CFU/g with the Δ*cbs* Δ*cse* strain; 1.7×10^4 CFU/g with the WT).

DISCUSSION

The observations that elevations in H_2S levels make both *E. coli* and *S. aureus* resistant to leukocyte-mediated killing and that decreases in bacterial H_2S levels increase the susceptibility of these bacteria to killing by the host both *in vivo* and *in vitro* demonstrate that H_2S can provide some protection of bacteria against early immune responses. This is further supported by the fact that *E. coli* and *S. aureus* utilize different enzymatic pathways for the synthesis of H_2S, and a loss of the respective enzyme activities in each bacterial species induces susceptibility to the host immune response. Our data show that the effects of bacterial H_2S are not likely due to effects on bacterial proliferation, as levels of growth of wild-type and H_2S-deficient *E. coli* and *S. aureus* were similar during the lag and exponential phases (Fig. 3 to 5). While specific mechanisms of protection are not known, previous reports provide some insight into possible bacterial defenses that could be regulated by H_2S. H_2S can prevent oxidative damage to bacteria through stimulation of superoxide dismutase and catalase activities, sequestration of free iron to prevent hydroxyl radical production via the Fenton reaction, and control of intracellular cysteine, which can stimulate hydroxyl radical production and inhibit electron transport at high levels (3, 4, 18). Additionally, hydrogen sulfide can inhibit the activity of myeloperoxidase (19). The increased susceptibility of opsonized H_2S-deficient bacteria to rapid killing by total leukocytes *in vitro* suggests that bacterial H_2S protects against rapid innate responses, which could include extracellular reactive oxygen species (ROS) and reactive nitrogen species (RNS) and intracellular ROS/RNS in phagosomes. This is supported by the susceptibility of H_2S-deficient *E. coli* to intracellular killing by macrophages, which is largely mediated by the generation of toxic radicals. However, the results from this study show significantly improved clearance of H_2S-deficient bacteria by total splenic leukocytes, of which the percentage of phagocytic cells is relatively low in the mouse. Therefore, there are likely other immune responses that may be resisted by bacterial H_2S, and further studies are needed to determine which specific immunological mechanisms can be resisted or avoided in the presence of bacterial H_2S. Therefore, although this study found that bacterial H_2S can protect bacteria from the host immune response, it is limited by a lack of identified mechanisms.

While nearly all bacterial species express orthologues of at least one mammalian H_2S-synthesizing enzyme (3), the relative importance of H_2S as a bacterial defense mechanism across a wide range of bacteria may vary and remains to be determined. Baseline levels of H_2S production vary between bacterial species, as demonstrated here (*S. aureus* < *E. coli*), and may further vary when bacteria are stressed by the host immune response. Additionally, the presence of different pathogenic and virulence factors that can affect the host response and bacterial infectivity may influence the relative importance of H_2S in bacterial self-defense. Nonetheless, as the two bacterial species used here differ not only in their pathogenicity and elicitation of specific host responses but also in their utilization of H_2S-producing enzymes, the results presented here implicate H_2S as a potentially global bacterial defense mechanism against the host immune response that may be targeted in the development of novel antimicrobial agents. Inhibition of bacterial H_2S may be a particularly beneficial approach to antimicrobial therapy in patients with inadequate immune functions, such as severe-burn patients. Burn injury induces impairments in both innate and acquired immune functions that can decrease the ability of the patient to respond effectively to an infection (20–22). The finding that both *E. coli* peritonitis and *S. aureus* burn wound infections are better controlled in burned mice when the respective bacteria are H_2S deficient

suggests that bacterial H_2S inhibition may have potential as a prophylactic measure to prevent infections in high-risk patients.

While the magnitude of the effects of H_2S deficiency on bacterial clearance suggests that H_2S inhibition alone would not be sufficient to treat an ongoing infection, it may be useful as an adjunct to increase the efficacy of antibiotics. H_2S can also provide resistance for many bacterial species against a broad range of antibiotics (3). Therefore, bacterial H_2S is implicated as a potential therapeutic target to enhance bacterial killing by both immune cells and antibiotics. However, the differential dependence of various bacterial species on the different H_2S-producing enzymes during the response to infection or antibiotic-induced stress mandates that the H_2S-inhibitory strategy be matched to the particulars of the H_2S-producing system in the respective bacterial strain(s). For example, the greater effect of PAG (than of AOAA) on the clearance of *S. aureus* (Fig. 2) suggests that *cse* activity may be more important for defense of *S. aureus* against the early immune response. Alternatively, combined treatment with multiple inhibitors that target all 3 primary bacterial H_2S-synthesizing enzymes may be more appropriate as a global treatment to be used prophylactically in high-risk patients or in conjunction with antibiotics. Unfortunately, the ability to further advance the current studies is restricted by a lack of inhibitors with specificity for the bacterial enzymes. While the mammalian enzyme inhibitors used here (Fig. 2) show some effects on bacterial H_2S production and susceptibility to leukocyte-mediated killing *in vitro*, the potency of each inhibitor against the mammalian homologues is higher (10). In mammals, H_2S is important for a wide range of important biological functions, including cellular bioenergetics and cardiovascular and neuronal functions (1). Additionally, H_2S has been reported to have both pro- and anti-inflammatory effects (23, 24). Therefore, these inhibitors are not suitable for the treatment of bacterial infections due to their inhibitory effects on host enzymes. Given the apparent role of bacterial H_2S in defense against both the immune response and antibiotics, there is a need for the development of inhibitors that are specific for bacterial H_2S-synthesizing enzymes to be considered novel antimicrobial agents.

MATERIALS AND METHODS

Bacterial strains. Two different wild-type species (and their H_2S mutant counterparts) that differ in their utilization of H_2S-producing enzymes were used. *E. coli* (MG1655) is an avirulent Gram-negative rod that primarily utilizes 3-MST, encoded by *sseA*, for H_2S production. To generate 3MST-deficient *E. coli* (Δ*sseA*), the *sseA* gene was excised from *E. coli* (3) by λInt/Xis site-specific recombination, using the pMWts-λInt/Xis-helper plasmid as described previously (25) and *sseA*-specific primer sequences (3). The *S. aureus* strain (USA300) is Gram positive and methicillin resistant and lacks 3MST but carries the *cbs-cse* operon (3). *S. aureus* wild-type and Δ*cbs* Δ*cse* strains were obtained from the Nebraska Transposon Mutants Library. Bacteria were grown in Luria-Bertani (LB) broth with shaking (200 rpm) at 37°C, and CFU were determined by plating diluted aliquots on LB agar plates. Growth rate curves were established by measuring the optical density at 600 nm (OD_{600}) over time. Culture medium for the Δ*cbs* Δ*cse* strain was supplemented with 10 μg/ml erythromycin. Bacterial growth and viability in cell culture medium (RPMI 1640 with 10% fetal bovine serum [FBS]) were measured by plating serial dilutions of cultures over time for determination of CFU per milliliter.

Animals. Male BALB/c mice (10 to 12 weeks of age; Envigo) were housed in a biosafety level 2 (BSL-2) animal facility under the supervision of the University of Texas Medical Branch (UTMB) Animal Resource Center and veterinarians, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Animal care and all procedures were compliant with NIH guidelines for the care and use of experimental animals and were approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee. Some mice received a full-thickness scald burn to approximately 35% of the body surface area, as described previously (13), under deep anesthesia (2% isoflurane) with preemptive analgesia (0.1 mg/kg of body weight buprenorphine). Fluid resuscitation (2 ml lactated Ringer's solution, i.p.) was administered after injury.

Bacterial clearance assays. (i) *In vitro*. For *in vitro* bacterial elimination assays, total leukocytes were isolated from the spleens of male BALB/c mice as described previously (26). Bacteria were opsonized by incubation with 5% mouse serum (Sigma-Aldrich) at room temperature for 15 min and then incubated at 37°C with leukocytes in RPMI 1640 supplemented with 10% FBS at a multiplicity of infection (MOI) of ~2 for 45 to 60 min. Back-plating of inocula was performed to confirm that all groups within an experiment received the same starting number of bacteria. To measure bacterial killing by macrophages, RAW 264.7 cells (American Type Culture Collection) were used and maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) at 37°C in a humidified incubator with 5% CO_2. Briefly, 1×10^5 RAW cells were seeded onto 13-mm coverslips in 24-well plates and allowed to adhere overnight. Nonadherent cells were removed with Hanks' balanced salt solution (HBSS), and bacteria were added at an MOI of ~5. Plates were centrifuged

at 500 × *g* and incubated at 37°C for 1 h. To enumerate total viable bacteria, 0.03% Triton X-100 was added, and serial dilutions were plated. Percent elimination was calculated as [($CFU_{time\ zero} - CFU_{final}$)/$CFU_{time\ zero}$] × 100. To enumerate viable intracellular bacteria, cells were washed twice with HBSS after 1 h of incubation with bacteria, treated with 100 μg/ml gentamicin (Sigma-Aldrich) for 30 min to kill extracellular bacteria, rinsed twice with HBSS, and incubated with 50 μg/ml gentamicin (time zero). After 0, 0.5, 1, and 3 h of incubation, cells were washed twice with HBSS and lysed with 0.3% Triton X-100, and serial dilutions were plated for determination of CFU. In some experiments, bacteria were cultured (8 to 12 h) in the presence of the following reagents (all reviewed in reference 10) prior to inoculation of leukocytes to manipulate bacterial levels of H_2S: the slow-release H_2S donor GYY4137 [morpholin-4-ium 4 methoxyphenyl(morpholino) phosphinodithioate] (0 to 1 mM; Sigma-Aldrich), the 3-MST inhibitor 2-[(4-hydroxy-6-methylpyrimidin-2-yl) sulfanyl]-1-(naphthalen-1-yl)ethan-1-one (0 to 10 μM; Molport), the CSE inhibitor D-L-propargylglycine (PAG) (0 to 30 mM; Sigma), or the CBS inhibitor amino-oxyacetic acid (AOAA) (0 to 1 mM; Sigma).

(ii) *In vivo*. To measure bacterial clearance *in vivo*, mice were injected i.p. with ~1×10^8 CFU *E. coli* or 5×10^8 CFU *S. aureus*, and tissues were harvested 12 to 16 h later. To measure bacterial growth and spread within burn wounds, 1×10^6 CFU *S. aureus* were injected under the upper half of the burn wound, and the lower half of the wound was harvested 24 h later. Inocula were back-plated to confirm doses. Tissues were homogenized in sterile saline, and serial dilutions were plated on agar. Data for all *in vivo* inoculation experiments shown are representative of results from 2 to 4 independently performed experiments. To assess systemic inflammation in response to infection, blood was collected when tissues were harvested for cultures, and IL-6 was measured by an enzyme-linked immunosorbent assay (ELISA) (Invitrogen, ThermoFisher).

Bacterial H_2S production. Lead acetate was used to detect and compare H_2S production by bacteria under different conditions, as described previously (3), with some modifications. Briefly, equal numbers of bacteria were cultured in 12-well plates at 37°C at 140 rpm overnight. The lid covering the culture plate had filter paper (Bio-Rad), saturated with 2% lead acetate (Sigma-Aldrich), affixed to the inside, above, but not in contact with, the bacterial cultures. H_2S reaction with lead acetate produces a brown lead sulfide stain that is visible on the filter paper (3).

Bacterial 3MST activity. Bacterial 3MST gene (*sseA*) (NCBI accession number NC_000913.3) was cloned and expressed in an *E. coli* vector system (pET43.1a) and purified (GenScript Inc., Piscataway, NJ, USA). The effect of the 3-MST inhibitor on the activity of the bacterial enzyme was determined as previously described (27), with modifications. Briefly, the inhibitor was added to assay buffer to yield final concentrations of 1 to 10 μM in a total assay mixture volume of 200 μl. The assay solution contained Tris HCl (50 mM; pH 8.0), bacterial full-length 3MST (*sseA*) (50 ng/well), the 3-MST substrate 3-mercaptopyruvate (100 μM final concentration), glutathione (2 μM final concentration), and the H_2S-specific fluorescent probe 7-azido-4-methylcoumarin (AzMc) (10 μM final concentration). The 96-well plates were incubated at 37°C for 2 h, and the increase in the AzMc fluorescence in each well was read at 450 nm (excitation wavelength [λ_{ex}] of 365 nm).

Statistical analysis. Data are presented as means ± standard errors of the means (SEM) and were analyzed using GraphPad Prism 7.0 for Windows. Multiple groups were compared by one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test, and two groups were compared by unpaired Student's *t* test. When unequal variances were detected by an F-test, data were log transformed for analyses. A *P* value of <0.05 was considered statistically significant.

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We declare that we have no conflict of interest.

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