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Bacterial Nitric Oxide Synthase Is Required for the *Staphylococcus aureus* Response to Heme Stress

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Abstract

Staphylococcus aureus is a pathogen that causes significant morbidity and mortality worldwide. Within the vertebrate host, *S. aureus* requires heme as a nutrient iron source and as a cofactor for multiple cellular processes. Although required for pathogenesis, excess heme is toxic. *S. aureus* employs a two-component system, the heme sensor system (HssRS), to sense and protect against heme toxicity. Upon activation, HssRS induces the expression of the heme-regulated transporter (HrtAB), an efflux pump that alleviates heme toxicity. The ability to sense and respond to heme is critical for the pathogenesis of numerous Gram-positive organisms, yet the mechanism of heme sensing remains unknown. Compound '3981 was identified in a high-throughput screen as an activator of staphylococcal HssRS that triggers HssRS independently of heme accumulation. '3981 is toxic to *S. aureus*; however, derivatives of '3981 were synthesized that lack toxicity while retaining HssRS activation, enabling the interrogation of the heme stress response without confounding toxic effects of the parent molecule. Using '3981 derivatives as probes of the heme stress response, numerous genes required for '3981-induced activation of HssRS were uncovered. Specifically, multiple genes involved in the production of nitric oxide were identified, including the gene encoding bacterial nitric oxide synthase (bNOS). bNOS protects *S. aureus* from oxidative stress imposed by heme. Taken together, this work identifies bNOS as crucial for the *S. aureus* heme stress response, providing evidence that nitric oxide synthesis and heme sensing are intertwined.

Graphical abstract

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ASSOCIATED CONTENT

The authors declare no competing financial interest.

Keywords

Staphylococcus aureus; heme stress response; nitric oxide synthase; small-molecule optimization

INTRODUCTION

Staphylococcus aureus is a Gram-positive pathogen that causes significant morbidity and mortality worldwide.^{1–4} Diseases caused by *S. aureus* range from superficial skin infections to invasive disease with considerable mortality. In this regard, *S. aureus* is a leading pathogen associated with life-threatening bloodstream infections.^{5,6} Furthermore, recent isolates exhibit significantly decreased susceptibility to the current antibacterial armamentarium.⁷ This has led to efforts focused on an increased understanding of the pathogenesis of this organism in order to identify novel therapeutic targets for the treatment of staphylococcal infection.

To colonize the vertebrate host, *S. aureus* requires numerous nutrients, such as heme.⁸ The heme requirement can be satisfied through two distinct mechanisms: importing exogenous heme through dedicated machinery and synthesizing endogenous heme de novo from metabolic precursors. Both heme acquisition and heme synthesis are required for full virulence of *S. aureus*.^{9,10} Once acquired, heme is used for various processes in the cell. Intact heme is utilized as a cofactor for enzymes including cytochromes in the electron transport chain, catalase for the detoxification of reactive oxygen species, and bacterial nitric oxide synthase (bNOS). Heme can also be employed as a nutrient iron source for bacteria through degradation by heme oxygenases to release free iron.¹¹

Although required to colonize the host, excess heme is toxic to bacteria. Thus, the ability to sense and detoxify heme is critical to many pathogens.¹² Specifically, multiple Gram-positive pathogens sense heme through the heme sensor system (HssRS) two-component

system (TCS).^{12,13} Upon activation, HssRS induces the expression of the heme-regulated transporter (*hrtAB*), which encodes an efflux pump that alleviates heme toxicity.^{13,14} The coordinated efforts of HssRS and HrtAB enable these pathogens to grow in the presence of high heme concentrations. The deletion of components of this pathway affects the virulence of *S. aureus*. Understanding the mechanism of HssRS activation will provide insight into the ability of pathogens to sense this required cofactor and survive within the hostile environment of the vertebrate host.

Recent studies have found that bacterial nitric oxide (NO) synthesis is important for bacterial survival in the host and suggest multiple roles for NO during bacterial infections.¹⁵ Bacterial pathogens encode genes that are similar to mammalian nitric oxide synthases, which led to the characterization of the bacterial nitric oxide synthase (bNOS) hemoprotein.¹⁶ The critical role of bNOS has been established in a number of organisms including *S. aureus* and *Bacillus anthracis*, where bNOS has been shown to protect against various antibiotics and oxidative stress.^{15,17–19} Considering its contribution to the virulence of these organisms, bNOS has been identified as a potential drug target for the treatment of infections.²⁰

We have previously reported a high-throughput screen (HTS) for activators of staphylococcal HssRS. This screen resulted in the identification of multiple chemical probes that activate HssRS.^{21,22} One such probe, VU0038882 (‘882), activates staphylococcal heme biosynthesis, providing a powerful tool for understanding heme biosynthesis in the context of heme sensing and infection.²¹ In this article, we describe the mechanism of HssRS activation by VU0043981 (‘3981), a second compound identified in the same HTS. Importantly, in contrast to ‘882, ‘3981 activates HssRS independently of heme accumulation, providing a unique probe for interrogating the heme sensing system. In this regard, a transposon screen utilizing a ‘3981-derived probe uncovered bNOS as crucial to the heme stress response, providing evidence that nitric oxide and heme sensing are linked in staphylococci.

RESULTS AND DISCUSSION

‘3981 Activates HssRS and Is Toxic to *S. aureus*.

A high-throughput screen (HTS) was performed to identify small-molecule activators of *S. aureus* HssRS.²¹ Compound VU0043981 (‘3981) was identified in this HTS as an activator of *S. aureus* HssRS (Figure 1A, inset). ‘3981 activates HssRS in a dose-dependent manner, and this activation is dependent upon the TCS (Figure 1A). These results demonstrate that ‘3981 is an activator of HssRS, leading to the activation of *hrtAB* expression in *S. aureus*.

Previously identified small-molecule activators of HssRS exhibit toxicity to fermenting *S. aureus*, although the mechanism of toxicity is yet to be determined.^{21,22} To probe the toxicity of ‘3981 to *S. aureus*, WT and two respiration-deficient strains, *hemB* and *menB*, were grown in the presence of increasing concentrations of ‘3981. *hemB* and *menB* strains are deficient in the production of heme and menaquinone, respectively, both of which are required for bacterial respiration.²³ Therefore, these strains must generate energy through fermentation. Compound ‘3981 is toxic to both WT and fermenting *S. aureus* strains

(Figure 1B). Taken together, these data suggest that '3981 activates HssRS and is toxic to fermenting and respiring *S. aureus*.

'3981 Activates HssRS Independently of Heme Accumulation.

Heme is the only identified activator of HssRS.^{12,13,21,24} To determine if '3981 activates HssRS through increasing endogenous heme biosynthesis, heme accumulation was quantified upon exposure to increasing concentrations of '3981 in the absence of exogenous heme. Heme accumulation was not observed in '3981-treated cells, suggesting that '3981 does not trigger HssRS through the activation of heme biosynthesis. This is in contrast to '882, a previously identified activator of HssRS that induces heme biosynthesis (Figure 2).²¹ Therefore, '3981 represents a small molecule that activates HssRS independently of heme accumulation, providing a unique tool to interrogate the heme sensing system in *S. aureus*.

'3981 Activity Requires Aerobic Respiration.

Although '3981 does not increase heme biosynthesis, heme could be required for the '3981-mediated activation of HssRS. Heme is utilized for numerous processes within the cell, including as a required cofactor for cytochromes and bacterial nitric oxide synthase.^{10,16,18,23} To test the requirement for heme biosynthesis in '3981-dependent HssRS activation, the activity of '3981 was measured in a heme biosynthesis mutant (*hemB*). Upon treatment of *hemB* with '3981, the activation of HssRS was not detected (Figure 3A). Because heme is required for respiration, the requirement for aerobic respiration in the activity of '3981 was interrogated. Respiration can be disrupted through either the inactivation of heme biosynthesis or by growing bacteria in an anaerobic environment in the absence of a terminal electron acceptor. *S. aureus* was treated with either '882 or '3981 during anaerobic growth. Whereas '882 activates HssRS in respiration-defective strains, '3981 does not (Figure 3B). To control for growth differences between strains, a plasmid constitutively expressing *xyIE* was included (*plgt.xyIE*). Taken together, these data suggest that '3981 does not activate heme biosynthesis, but its mechanism of action requires aerobic respiration.

Structure–Activity Relationship Studies Identify '3981 Derivatives with Increased Activity.

The general toxicity of '3981 toward *S. aureus* hindered its use as a probe of heme sensing. Using this structure as a starting point, a library of derivatives was generated to determine structure–activity relationships (SAR, Supporting Information Table 3) and to identify compounds exhibiting increased HssRS activation (Figure 4A). The 4-biaryl ether system was determined to be required for HssRS activation because replacement with smaller biaryl, aryl, or aliphatic groups resulted in a loss of activity. The benzylic carbon is also required for activity because the replacement of the 4-fluorobenzyl with a 4-fluorophenyl group resulted in an inactive compound. Several derivatives were synthesized with various substituents on the benzylamine moiety. Removal of the electron-withdrawing 4-fluorine and substitution with an electron-rich methyl ether at the 3-position resulted in a compound, VU0047501 ('7501), with 4 times the activity of the parent molecule (Figure 4A,B). In addition to increased HssRS activation, '7501 was considerably less toxic than '3981 (Figure 4C). Furthermore, '7501 retained identical phenotypes to '3981 with regard to an inability to induce heme accumulation and a requirement for aerobic respiration (Supporting

Information Figure 1). Because of the increased activity and lack of toxicity, '7501 was utilized to probe the heme stress response without confounding effects as a result of the toxicity of the parent molecule.

Transposon Screen Identifies Bacterial Nitric Oxide Synthase As Required for the Heme Stress Response.

'7501 exhibits increased activation of HssRS relative to '3981; therefore, the ability of '7501 to adapt *S. aureus* to heme toxicity was tested.^{12,21} Indeed, '7501 adapts *S. aureus* to the toxic effects of heme. This result implies that HssRS activation was sufficient to upregulate HrtAB expression to enable the bacteria to subsequently cope with toxic concentrations of heme (Figure 5A). Using the '7501-dependent heme adaptation as an assay platform, the Nebraska Transposon Mutant Library, consisting of approximately 2000 strains of *S. aureus* with single, defined inactivating mutations, was screened to identify genes required for '7501 activity. Multiple genes were identified that are required for the ability of '7501 to activate the HssRS-HrtAB heme stress response (Supporting Information Table 4). Importantly, a number of these genes, including the gene encoding cytochrome protein Qox, are required for respiration, consistent with data presented in Figure 2 (Supporting Information Table 4). In addition, a number of the identified genes are in the pathway for the production of NO directly through the inactivation of bNOS or for enzymes involved in pathways leading to NO formation (Figure 5B). This suggests that NO synthesis and the bacterial heme stress response are linked within *S. aureus*. Heme serves as a cofactor for bNOS, implying the possibility of a functional link between these two systems.

To verify that bNOS is required for the ability of *S. aureus* to adapt to heme stress, the *nos::ermC* mutation was transduced into *S. aureus* strain Newman (*nos*). As expected, the interruption of *bNOS* led to decreased resistance to heme toxicity (Figure 6). Furthermore, this decreased resistance of *nos* to heme can be restored by providing a full-length copy of *nos in trans* (Figure 6). Taken together, these results demonstrate that bNOS is required for adaptation to heme toxicity.

bNOS Contributes to Heme Sensing and Detoxification Independently of *hrtAB*.

The mechanism by which bNOS protects *S. aureus* from heme toxicity could be due to the activation of HssRS or general protection from heme toxicity through an HssRS-independent mechanism. To determine if bNOS contributes to heme sensing through HssRS, WT and *nos* were treated with either vehicle, heme, or '7501 and the response of the *hrt* reporter was measured as described above. Interestingly, *nos* exhibits a significant decrease in the activation of the *hrt* promoter; however, the magnitude of this change does not fully account for the differences in response to heme (Figure 7A). On the basis of these observations, we hypothesized that in addition to an involvement in sensing heme toxicity, bNOS is involved in resisting heme toxicity in a mechanism distinct from HssRS activity. To test this hypothesis, an *hrtB/bNOS* double mutant was constructed to determine if this strain is more sensitive to heme toxicity than the inactivation of *hrtB* alone. A strain lacking the heme efflux system (*hrtB*) is more resistant to heme toxicity than an *hrtB/bNOS* double mutant, implicating bNOS in protecting *S. aureus* from heme toxicity independently of *hrtB* (Figure 7B). Taken together, these data suggest that bNOS both affects the sensing of heme by

HssRS and protects *S. aureus* from heme toxicity through a mechanism distinct from the actions of HssRS and HrtAB.

CONCLUSIONS

Maintaining correct heme homeostasis is crucial for the survival of bacterial pathogens within the host.^{12,25} Heme is a required molecule for many processes within the cell; however, it is also toxic to the organism. This concept has been termed the heme paradox.²⁵ Therefore, being able to sense and respond to increased heme levels is vital for organisms to survive in the diverse environments experienced within the host. Understanding the mechanisms by which these bacterial pathogens sense and respond to heme stress has the potential to uncover novel targets for therapeutic development because altering the ability of *S. aureus* to sense heme affects the virulence of this organism.¹²

Understanding the specific requirements of TCS activation remains a challenge.²⁶ To further our understanding of the heme stress response and the mechanisms of HssRS activation, this work utilized a small-molecule activator of the heme sensing system. Previously identified small-molecule activators of HssRS have multiple activities within the cell and have provided a more thorough understanding of the mechanisms behind heme homeostasis within the bacterial cell.^{21,22} Consistent with this, we report here that '3981 is both toxic to *S. aureus* and activates HssRS in a respiration-dependent manner (Figures 1 and 3). Structure–activity relationship studies have separated these activities (Figure 4), thereby providing '3981 derivatives that lack toxicity, thus enhancing the ability of this scaffold to interrogate the heme stress response.

Heme is the canonical activator of HssS in *S. aureus*.^{12,13,21} Small-molecule activators provide an opportunity to interrogate TCS activation. Importantly, '3981 does not induce heme accumulation within *S. aureus* (Figure 2). These data support the possibility that there may be multiple ligands required for HssS activation. Activation by multiple ligands is not unprecedented. In *Mycobacterium tuberculosis*, DosS and DosT are histidine kinases that sense heme, and their activity is modulated by other ligands, including CO, NO, and O₂.²⁷ This leads to the exciting hypothesis that perhaps HssS senses heme in complex with another intracellular molecule to modulate the response to heme stress.

Previous work has shown the importance of bNOS in survival in the host as well as resistance to various antibiotics and oxidative stress.¹⁸ The importance of endogenous nitric oxide production has been demonstrated in numerous organisms, including pathogens *S. aureus* and *B. anthracis*.¹⁸ Taken together, bNOS has been proposed as a viable antibacterial target.²⁰ Consistent with this, utilizing '3981 has identified another role for bNOS in *S. aureus*, specifically in resistance to heme stress. Although bNOS contributes slightly to heme sensing, it plays a crucial role in resisting heme stress independently of the HssRS–HrtAB heme stress response (Figure 7). Heme induces significant oxidative stress in *S. aureus*.²⁸ It is likely that bNOS protects *S. aureus* from the oxidative stress imposed by heme because bNOS protects bacteria from oxidative stress. Targeting bNOS for antibiotic development has the potential to affect a number of systems within the bacteria and therefore significantly impact the virulence of this pathogen.

Overall, this work has provided a unique look into the heme stress response in *S. aureus*. SAR separated two phenotypes induced by '3981, ultimately providing probes with a significantly increased ability to activate the bacterial heme stress response. In addition, these probes activate the heme sensing system independently of heme accumulation, suggesting that heme may not be the only molecule required for HssRS activation. Finally, these probes have identified bNOS as required for the heme stress response, uncovering another important role of nitric oxide synthesis in bacterial pathogens and highlighting bNOS as a target for antibacterial development.

METHODS

Bacterial Strains, Growth Conditions, and Plasmids.

Cloning was performed in *Escherichia coli* DH5a (Invitrogen). *S. aureus* strains were grown in tryptic soy broth (TSB) or agar (TSA), and *E. coli* was grown in lysogeny broth (LB) or agar (LBA). The strains and plasmids used in this study are described in Supporting Information Tables 1 and 2, respectively.^{33–37}

S. aureus strain allele *nos::ermC* was described previously and was transduced into Newman using the Φ –85 bacteriophage (creating *nos*) or strain *hrtB* (creating *hrtB/ nos*).^{29,30}

A complementation construct for *nos* was created by PCR-amplifying *nos* using Newman genomic DNA as a template and cloning into pOS1.p/*gt*.

Promoter Activity Assay.

The promoter activity assay was performed as previously described.¹³ Briefly, *phrAB.XylE* was electroporated into strain *nos*. The other strains that were used have been previously described.²¹ Overnight cultures were diluted 1:100 into TSB supplemented with 10 μ g/mL chloramphenicol and grown for 24 h and assayed as previously described.¹² The experiment was performed in triplicate on three separate days ($n = 9$). Data are presented as mean $\pm$ SEM. The Student's *t* test was performed to determine the significance.

IC₅₀ Determination.

To determine the toxicity of the compounds, *S. aureus* overnight cultures were diluted 1:100 in a 96-well plate containing 200 μ L of TSB with or without compound supplementation. Cultures were grown at 37 °C for 7 h (stationary phase of untreated bacteria), and the OD₆₀₀ was determined. Data were transformed to the percent growth of untreated bacteria, and compound concentrations were log transformed and fitted using GraphPad (Prism).

Heme Quantification.

The quantification of intracellular heme was performed as previously described.²¹ The experiment was performed in triplicate on three separate days ($n = 9$). Data are presented as the mean $\pm$ SEM. The Student's *t* test was performed to determine the significance.

Chemical Synthesis.

Synthesis procedures and compound characterization data can be found in the Supporting Information.³²

Heme Adaptation.

Heme adaptation assays were performed similarly to those previously described.^{12,21} The assay was performed in 96-well plates with pretreatment with vehicle (DMSO), heme, or a '3981-derived compound. Briefly, colonies were selected into 150 μ L of TSB in a 96-well plate and grown for 6 h with shaking at 37 °C. Cultures were subcultured 1:150 under the condition being tested and grown overnight. In the morning, cultures were subcultured again 1:150 into the heme concentration being interrogated or the vehicle control and were grown for 6 h. OD₆₀₀ data were determined and compared. The experiment was performed in triplicate on three separate days ($n = 9$). Data are presented as the mean $\pm$ SEM. The Student's t test was performed to determine the significance.

Transposon Mutant Screen.

The staphylococcal Nebraska Transposon Mutant Library was screened using the protocol described above.³¹ Cultures were pretreated with '7501 overnight, and subcultured into 50 μ M heme. Hits were identified as those that were greater than 2 standard deviations below the mean growth of the plate being tested.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acsinfecdis.6b00081](https://doi.org/10.1021/acsinfecdis.6b00081). Chemical synthesis and supplemental figures and tables (PDF)

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Figure 1.

'3981 activates HssRS and is toxic to *S. aureus*. (A) Compound '3981 was identified in an HTS for activators of HssRS. *S. aureus* WT and *hssRS* harboring *phrt.xylE* were treated with increasing concentrations of '3981. '3981 activates HssRS in a dose-dependent manner requiring the HssRS two-component system. (Inset) Structure of '3981. * denotes $p < 0.05$, ** denotes $p < 0.0001$. # denotes data below the limit of detection. (B) WT, *hemB*, and *menB* were treated with increasing concentrations of '3981. '3981 inhibits the growth of *S. aureus*.

Figure 2.

'3981 does not increase endogenous heme biosynthesis. *S. aureus* was grown in the presence of '882 and '3981 and in the absence of any exogenous heme source. '882 activates heme biosynthesis, leading to heme accumulation.²¹ '3981 does not activate heme biosynthesis, suggesting that '3981 activates HssRS through a mechanism distinct from increased heme production. Significance was determined in comparison to vehicle treatment. * denotes $p < 0.05$; ** denotes $p < 0.0001$.

Figure 3.

'3981 activity is dependent on aerobic respiration. (A) To determine the requirement of heme synthesis for HssRS activation, *S. aureus* WT and *hemB* harboring *phrt.xylE* were treated with increasing concentrations of '3981. HssRS activation was not seen in the *hemB* strain. Significance was determined in comparison to the vehicle treatment of *phrt.xylE*. (B) Because heme is required for many cellular processes, including respiration, the ability of '3981 to activate HssRS in an anaerobic environment was tested. WT harboring *phrt.xylE* was treated with heme, '882, or '3981. '3981 activity is dependent upon intact respiration. * denotes $p < 0.05$; ** denotes $p < 0.0001$. # denotes data below the limit of detection.

Figure 4.

'3981 derivatives were identified with increased HssRS activation and decreased toxicity. (A) Systematic modification identified important characteristics of '3981, leading to the identification of compound '7501 with an increased ability to activate HssRS while lacking toxicity of the parent compound. (B) WT harboring *phr.t.xylE* was treated with equivalent concentrations of '3981 and '7501. '7501 shows 4-fold increased activity compared to that of the parent molecule. * denotes $p < 0.05$; ** denotes $p < 0.0001$. (C) Growth inhibition of

'3981 and '7501 was determined. Whereas '3981 inhibits the growth of WT bacteria, '7501 lacks toxicity.

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Figure 5.

Adaptation screen uncovers the importance of nitric oxide to the heme stress response. (A) *S. aureus* strain USA300 JE2 was pretreated with '7501 followed by exposure to toxic concentrations of heme. '7501 adapts *S. aureus* to heme toxicity. (B) The Nebraska Transposon Mutant Library was screened to identify strains that could not be adapted to heme toxicity using '7501. Many strains disrupted in genes encoding nitric oxide synthesis proteins were identified (gray, bold).

Figure 6.

Bacterial nitric oxide synthase is required for adaptation to heme toxicity. *S. aureus* WT harboring pOS1.*Plgt*, *nos* harboring pOS1.*Plgt*, and *nos* harboring *plgt.nos* were pretreated with heme, followed by exposure to toxic concentrations of heme. WT is adapted to the toxic effects of heme, whereas *nos* is significantly impaired when treated with toxic concentrations of heme. This phenotype is complemented by the addition of *nos* expressed in trans. Data shown are a representative sample. * denotes $p < 0.05$.

Figure 7.

Bacterial nitric oxide synthase affects heme sensing and protects *S. aureus* from heme toxicity independently of *hrtAB*. (A) To determine if bNOS contributes to heme sensing, *S. aureus* WT and *bnos* harboring *phr.xylE* were treated with heme. The *bnos* strain shows a significant decrease in the ability to sense heme, suggesting that bNOS plays a role in heme sensing in *S. aureus*. (B) To determine if bNOS contributes to protection from heme toxicity independently of HssRS/HrtAB, strains WT, *bnos*, *hrtB*, and *bnos/ hrtB* were grown in the presence of low concentrations of heme. Strain *bnos/ hrtB* has a significant growth defect compared to *hrtB*, suggesting that bNOS contributes to the protection from heme toxicity independently of HssRS/HrtAB. * denotes $p < 0.05$; ** denotes $p < 0.0001$.