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An Essential Role for Bacterial Nitric Oxide Synthase in *Staphylococcus aureus* Electron Transfer and Colonisation

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Abstract

Nitric oxide (NO $\cdot$) is a ubiquitous molecular mediator in biology. Many signaling actions of NO $\cdot$ generated by mammalian NO $\cdot$ synthase (NOS) result from targeting of the haem moiety of soluble guanylate cyclase. Some pathogenic and environmental bacteria also produce a NOS that is evolutionary related to the mammalian enzymes, but a bacterial haem-containing receptor for endogenous enzymatically-generated NO $\cdot$ has not been previously identified. Here we show that NOS of the human pathogen *Staphylococcus aureus*, in concert with an NO $\cdot$ -metabolising flavohaemoprotein, regulates electron transfer by targeting haem-containing cytochrome oxidases under microaerobic conditions to maintain membrane bioenergetics. This process is essential for staphylococcal nasal colonisation and resistance to the membrane-targeting antibiotic daptomycin, and demonstrates the conservation of NOS-derived NO $\cdot$ -haem receptor signaling between bacteria and mammals.

INTRODUCTION

Nitric oxide (NO $\cdot$) plays a central role in biological processes ranging from signal transduction to vasorelaxation, neurotransmission and cell death¹. Many signaling actions of NO $\cdot$ result from its reversible binding to the haem moiety of soluble guanylate cyclase, leading to an increase in cyclic GMP levels and diverse downstream events². Mammalian cells produce NO $\cdot$ from L-arginine via one of three NO $\cdot$ synthase (NOS) isoforms³. Some bacteria, notably Firmicutes, Actinobacteria and Deinococci, also possess a NOS which is closely related to the oxygenase domain of mammalian NOS but must partner with a separate reductase that serves as an electron donor^{4,5}. Although specialised roles of bacterial NOS have been proposed in nitration reactions⁶ or resistance to oxidative stress^{7,8}, neither a

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AUTHOR CONTRIBUTIONS

Conceptualization, TLK, and FCF; Methodology, TLK, SRM, JMP, SJL, and FCF; Investigation, TLK, SRM, DVT, ES, SJL, and FCF; Writing- Original Draft, TLK and FCF; Writing- Review & Editing, TLK, SRM, JMP, SJL, and FCF; Funding Acquisition, FCF.

general physiological function for bacterial NOS nor a haem-target for endogenous enzymatically-derived NO $\cdot$ in bacteria has been identified.

One NOS-producing bacterial species is *Staphylococcus aureus*, an important human pathogen that colonises an estimated two billion persons worldwide and has become a leading cause of cutaneous, respiratory and bloodstream infections⁹. Deaths from methicillin-resistant *S. aureus* (MRSA) now exceed those caused by Human Immunodeficiency Virus (HIV) in the United States¹⁰. The *S. aureus* NOS (saNOS) enzyme has been implicated in phenotypes associated with virulence, including reduced survival in murine cutaneous and renal abscess models of infection and increased sensitivity to oxidative stress and antibiotics, but the molecular mechanism by which endogenous NO $\cdot$ enhances staphylococcal virulence is unknown^{11,12}.

We have recently shown that the SrrAB two-component regulatory system (TCS) is essential for *S. aureus* virulence and biofilm formation, and activates gene expression in response to NO $\cdot$ or to microaerobic conditions^{13,14}. The responsiveness of the SrrAB TCS to both NO $\cdot$ and hypoxia appears to result from the effects of these environmental conditions on the redox state of the menaquinone (MK) pool. The further investigation of NO $\cdot$ signaling in *S. aureus* led us to examine the effects of endogenous NO $\cdot$ on staphylococcal physiology and host interactions.

RESULTS

Staphylococcal NOS-derived NO $\cdot$ Controls the Rate of Aerobic Respiration

As the terminal oxidases of bacteria are susceptible to inhibition by exogenous NO $\cdot$ ^{15,16}, we hypothesized that endogenous NO $\cdot$ may also act by targeting the haem centers of the cytochromes. *S. aureus* O $_2$ consumption in the presence or absence of saNOS was monitored using a Clark-type electrode immersed in a bacterial cell suspension. An *saNOS*-deficient mutant was found to consume O $_2$ more rapidly than an isogenic wild-type parent strain (fig. 1a, and Supplementary figure 1a). Conversely, an *hmp* mutant strain, which is impaired in detoxifying NO $\cdot$, consumed O $_2$ more slowly than wild-type. The effect of endogenous NO $\cdot$ on O $_2$ consumption was most pronounced at low O $_2$ concentrations. Accelerated oxygen consumption could be phenocopied in wild-type bacteria by addition of the NOS inhibitor L-NAME (L-N^G-nitroarginine methyl ester) in a dose-dependent fashion, which had no effect on the *saNOS* mutant (fig. 1b and Supplementary figure 1b). The effect of saNOS on O $_2$ consumption was confirmed after the construction of *saNOS* mutations in the unrelated *S. aureus* RN4220 and UAMS-1 strains (Supplementary figure 1c), indicating that the regulation of O $_2$ consumption is a conserved property of saNOS. In addition, a plasmid carrying *saNOS* was constructed to complement the *saNOS* mutation and restored the rate of O $_2$ consumption back to wild-type levels (Supplementary figure 1d). Similarly, an isogenic *bsNOS* mutant⁷ of *Bacillus subtilis* strain 168 exhibited enhanced O $_2$ consumption relative to its wild-type parent (Supplementary figure 1e and f).

Endogenous NO $\cdot$ Reduces the Cellular Menaquinone (MK) Pool

Inhibition of the terminal cytochrome oxidases is predicted to reduce the pool of MK, the sole quinone electron carrier in *S. aureus*. As a measure of MK reduction¹³, expression of the SrrAB-regulated *pflB*, *hmp* and *nrdG* genes was monitored by qRT-PCR (fig. 2a). Impaired activation of SrrAB-activated genes was observed in an *saNOS*-deficient mutant, consistent with reduction of the MK pool under microaerobic conditions as a result of the inhibition of haem-containing terminal oxidases by saNOS-generated NO $\cdot$.

saNOS Regulates the Transition from Aerobic to Nitrate Respiration

MK serves as the electron carrier for both aerobic and nitrate (NO $_3^-$) respiration in *S. aureus*. NO $_3^-$ consumption was monitored in parallel to O $_2$ consumption in late logarithmic cultures (fig. 2b). Culture medium was supplemented with NaNO $_3$ at concentrations comparable to those found in body fluids¹⁷. NO $_3^-$ reduction was observed to initiate in wild-type *S. aureus* prior to the exhaustion of O $_2$ availability. However, NO $_3^-$ consumption was delayed in an *saNOS* mutant until nearly all O $_2$ had been consumed (fig. 2b). The disappearance of NO $_3^-$ was confirmed to be a consequence of NO $_3^-$ reduction, as NO $_3^-$ consumption was abolished in a *narG* mutant lacking the respiratory membrane-bound NO $_3^-$ reductase (Supplementary figure 2a). No differences in the expression of *narG* were observed in wild-type and *saNOS* mutant *S. aureus* (Supplementary figure 2b). In addition, an *srrAB* mutant exhibited earlier initiation of NO $_3^-$ respiration (Supplementary figure 2c), possibly as a result of reduced haem and cytochrome biosynthetic gene expression¹³. Complementation of the *saNOS* mutant restored timely NO $_3^-$ consumption, although *saNOS* expression from a plasmid led to a delay in growth, most likely as a consequence of increased endogenous NO $\cdot$ production (Supplementary figure 2c).

NO $_3^-$ reduction results in the generation of nitrite (NO $_2^-$), which is excreted until available NO $_3^-$ is exhausted, at which time the NO $_2^-$ is transported back into the cell and reduced to ammonia¹⁸. Despite a significant lag in NO $_3^-$ respiration, *saNOS* mutants rapidly consume NO $_2^-$ as it is generated by the reduction of NO $_3^-$ (Supplementary figure 2a). The consumption of NO $_3^-$ and NO $_2^-$ was observed to be dependent on NarG and NirB, respectively (Supplementary figure 2a).

Bacterial NOS Maintains Membrane Potential (ψ) During Microaerobiasis

As both aerobic and nitrate respiration are electrogenic, the regulation of electron transfer by NO $\cdot$ is anticipated to have important bioenergetic implications. Membrane potential (ψ) was therefore monitored by a ratiometric method using the dye DiOC $_2$ (3) (3,3'-diethyloxacarbocyanine iodide)^{19,20}. Although ψ was observed to decline in both wild-type and *saNOS*-deficient cells as O $_2$ becomes limiting (fig. 3a and b), only the wild-type cells maintained a reduced membrane potential, whereas dissipation of the ψ was observed in the *saNOS* mutant. Although the *saNOS* mutant eventually consumes NO $_3^-$ (Supplementary figure 3a, 3c), ψ was not restored to wild-type levels. Acetate production was measured as an indicator of glycolytic flux and TCA cycle activity in the presence or absence of *saNOS*²¹. Comparable levels of acetate were produced by wild-type and *saNOS* mutant *S. aureus* throughout growth (Supplementary figure 3b). Monitoring of intracellular redox balance revealed only a modest increase in NAD $^+$ in an *saNOS* mutant during early

exponential phase ($P < 0.05$; 3.5 hr time point) in the presence or absence of $100 \mu\text{M NaNO}_3$ (Supplementary figure 3d). This is consistent with the observed increase in oxygen consumption and a concomitant increase in NADH oxidation in the absence of endogenously produced NO . During the transition to stationary phase, an *saNOS* mutant also exhibits a slight increase in NAD^+ and a concomitant fall in NADH ($P < 0.005$; 7.25 hr time point; Supplementary figure 3d), attributable to delayed nitrate respiration (Supplementary figure 3c) leading a decline in ψ in the *saNOS* mutant and an increased dependence on fermentation²².

Endogenous NO Protects *S. aureus* from Daptomycin

The cyclic lipopeptide antibiotic daptomycin is an important agent used for the treatment of resistant *S. aureus* infections that has been shown to target cell membranes to result in membrane depolarization^{23,24}. Previous studies have shown that *saNOS* mutants are more susceptible to daptomycin¹², and accordingly we observed the more rapid recovery of wild-type *S. aureus* after daptomycin treatment in comparison to an *saNOS* mutant (fig. 4a and Supplementary figure 4a). Importantly, we found that NO_3^- supplementation enhanced the recovery of wild-type cells but did not affect *saNOS* or *narG*-deficient strains, consistent with a mechanism dependent on NO_3^- reduction. Furthermore, when ψ was assessed in terminal samples 16 hrs post-daptomycin treatment, wild-type *S. aureus* grown in the presence of NO_3^- were still able to generate ψ , whereas the *saNOS* and *narG* mutant strains exhibited the complete dissipation of ψ (fig. 4b and Supplementary figure 4b).

Endogenous NO is Required for *S. aureus* Nasal Colonisation

Finally, to assess the biological relevance of saNOS, we developed a novel murine model of staphylococcal colonisation of the nose, the primary site of colonisation in humans. Streptomycin was administered to mice for two weeks to eliminate the presence of the murine commensal bacterium *Staphylococcus xylosus*. A 1:1 mixture of wild-type and *saNOS* mutant *S. aureus* or the plasmid-complemented *saNOS* mutant was subsequently administered intranasally to anaesthetised mice by micropipette. Colonised animals were serially sampled at 24–48h intervals to determine the competitive index (fig. 5). The absence of saNOS was found to transiently enhance colonisation, possibly due to increased aerobic respiration, but resulted in dramatically impaired colonisation by day six. Normal levels of colonisation were restored by the cloned *saNOS* gene on a plasmid. Defective colonisation by the *saNOS* mutant persisted in mice lacking *iNOS*, indicating that the biological role of bacterial NOS is not to stimulate resistance to exogenous nitrosative stress.

DISCUSSION

Most interest in NO biosynthesis during infection has focused on the antimicrobial actions of host-derived NO generated by inducible isoform of mammalian NOS²⁵. However, it has been less well appreciated that many bacteria also possess functional NOS enzymes⁵, and bacterial NO biosynthesis appears to play a role in the virulence of the human pathogen *Staphylococcus aureus*^{11,12}, although the mechanism by which bacterial NOS contributes to pathogenesis has not been established. In the present study, we demonstrate a mechanism by

which saNOS regulates electron transfer and energy generation to maintain the membrane potential under O₂-limited conditions.

High concentrations of exogenously administered NO[•] can inhibit aerobic respiration in *S. aureus*^{13,22} as a result of reversible interaction with the haem centers of the Cyt aa₃ and Cyt bd terminal oxidases^{15,16,26,27}. As both NO[•] generation by saNOS and NO[•] consumption by the Hmp flavohaemoprotein are O₂-dependent, saNOS and Hmp in concert provide a homeostatic mechanism to regulate membrane electron transfer in response to varying O₂ availability^{16,28} (fig. 6). The present observations demonstrate for the first time that the low levels of NO[•] produced endogenously by saNOS^{11,12} are sufficient to inhibit aerobic respiration. Cytochrome oxidase inhibition by endogenous NO[•] diverts electrons from the MK pool to nitrate reductase, which maintains the membrane potential (ψ) under microaerobic conditions. The K_m of various NOS isoforms for O₂ range from 6–23 μ M²⁸, which is substantially higher than the K_m of cytochrome oxidases for O₂²⁹ but lower than that of Hmp³⁰, is consistent with this model, which may be seen as analogous to the actions of mammalian eNOS in the regulation of mitochondrial oxygen consumption³¹ and the mitochondrial membrane potential³².

In eukaryotes, the haem moiety of soluble guanylate cyclase is a key target of NO[•] in signal transduction². Here we show that NOS-NO[•]-haemoprotein interactions also play a crucial signaling role in bacteria. NO[•] produced by saNOS targets haem-containing terminal oxidases to allow bacteria to adapt to microaerobic conditions. Thus, NOS regulates electron transfer to optimize membrane bioenergetics. This action is required for the ability of *S. aureus* to resist the actions of daptomycin, an antibiotic that targets the cell membrane to depolarise the membrane potential^{23,24}, and is also essential for staphylococcal colonisation of a mammalian host. The pharmacological inhibition of bacterial NOS^{33,34} may therefore provide a novel strategy to eliminate staphylococcal colonisation and prevent infections.

As bacterial NOS is evolutionarily related to the mammalian enzymes⁴, and in view of mounting evidence that endogenous NO[•] regulates mitochondrial respiration³², the regulation of electron transfer by targeting the haem moiety of cytochrome oxidase may represent the primordial biological function of NOS. It should be noted that some bacteria contain proteins with H-NOX domains that are able to sense NO[•]³⁵. However, with rare exception³⁶, H-NOX and NOS proteins are not found within the same bacteria. In contrast, bacteria that produce NOS also possess a respiratory electron transport chain. In view of the diverse pathogenic and environmental bacteria that produce NOS³⁷ and the ubiquity of microaerobic environments in nature³⁸, the contribution of NOS to bacterial physiology during conditions of O₂ limitation is likely to be widespread.

MATERIALS AND METHODS

Strain construction

Strains, plasmids and primers are listed in Table S1. The methicillin-resistant *Staphylococcus aureus* strain COL was used as the primary strain for all experiments. An *saNOS* mutation was generated as previously described¹¹, and plasmid TR27 was transduced into strains COL, RN4220, and UAMS-1 to generate FLS092, FLS088 and

FLS340, respectively. COL strains with *narG* mutation was generated using primer pairs *narG* -L and R, and *narG* -L1 and R1 (Supplemental table 1), to generate fragments for overlapping PCR before blunt-ligation into *EcoRV*-digested pIMAY to generate plasmids pTK7, which was purified in *E. coli* and electroporated into *S. aureus* strain RN4220. Plasmid pTK7 was transduced into *S. aureus* COL and the isogenic *saNOS* mutant using phage phi-11 and used for allelic replacement³⁹. The *narG* mutation was confirmed by PCR with primer sets *narG* -L3 and R4, and *narG* -L4 and R4, respectively.

Measurement of O₂ consumption

Cells were grown in Tryptic Soy Broth (TSB) to OD₆₆₀ 1.0 prior to measurement of O₂ consumption. Cells were then pelleted and resuspended in 1mL PBS for every 25mL of cells that were pelleted. An apparatus to monitor O₂ consumption was set up as follows: a hose was connected to an air nozzle, which was further connected via a filtered pipet tip to an autoclave-sterilised tube that was anchored to a 50mL beaker on a magnetic stir plate in a temperature-controlled room (37°C). A Clark dip-type O₂ electrode (Microelectrodes, Inc.) was immersed in 24mL PBS. The air nozzle was turned on and kept constant throughout each experiment, and a secondary connection between the tubes was used to disconnect from the airflow. A small stir bar was placed in the beaker and 0.1% glucose (final concentration) was added to the beaker, and the O₂ levels were allowed to equilibrate for 30 sec prior to the addition of 1mL of the cell resuspension. Thirty sec after the addition of the cell suspension, the airflow tubing was disconnected and the sample monitored until O₂ consumption was complete. To perform the experiments utilising the NOS inhibitor L-N^G-nitroarginine methyl ester (L-NAME, Sigma), the protocol was modified as follows: the cell suspension was added to 24mL of PBS along with various concentrations of L-NAME, the mixture was allowed to equilibrate for 1.5 min prior to the addition of 0.1% glucose to stimulate respiration, and the airflow hose was disconnected after 30 sec. A recorder (ADInstruments) was connected to the O₂-probe via an adaptor. Data were collected using Lab Chart software (ADInstruments) and analyzed with GraphPad Prism software. The following equation was used to convert %O₂ into mM values: Solubility of O₂ in mM= [(a/22.414) × (760 – p)/760 × (r%/100)] × 1000, with a = absorption coefficient at 37°C = 0.02384, and p = vapor pressure of water at 37°C = 47.1. Data were analysed using a one-phase decay non-linear regression model, with shared plateau values and y₀ constrained to 0.2075.

Quantitative real-time RT-PCR

Steady-state mRNA levels were measured in WT or *saNOS* mutant *S. aureus* grown to OD₆₆₀ 0.5, 1.0 or 2.0 in TSB. RNA isolation was performed using the RNAeasy Miniprep kit (Qiagen) with an additional lysis step using Lysostaphin (AMBI). RNA was converted to cDNA using the Quantitect Reverse Transcription kit (Qiagen). Quantitative real-time RT-PCR analysis was performed using a BioRAD CFX96 Real-Time System with QuantiFast SYBR Green Mix (Qiagen). Four biological samples were assayed in triplicate with the primers listed in Supplementary Table 1. Data and statistical analysis were performed using GraphPad Prism software.

Simultaneous measurement of O₂ and NO₃⁻ consumption

To simultaneously assay O₂ and nitrate (NO₃⁻) consumption, *S. aureus* WT or mutant derivatives were grown in TSB with 50μM NaNO₃ in flasks to an OD₆₆₀ of 0.5. One-hundred mL of culture were transferred to a sterile dish on a magnetic stir plate in a temperature-controlled room (37°C). Sterile tubing was connected to an air nozzle with a filtered tip separating the sections, and air was continuously bubbled through the culture. As previously, a dip-type Clark electrode was used to monitor O₂ levels, while 1mL samples were obtained every 5 min over a 2 hr period for OD₆₆₀ determinations and the measurement of NO₃⁻ performed via the Griess reaction as previously described⁴¹. Briefly, after OD₆₆₀ readings, samples were pelleted and the supernatant transferred to a new tube. Tubes were incubated at 65°C to kill remaining bacterial cells. Fifty μL of supernatant were mixed with 50μL nitrate reductase buffer composed of the following: 0.056M K₂HPO₄, pH7.5; 2.5μM FAD and 100μM NADPH, with or without 0.06U/mL nitrate reductase from *Aspergillus* (all from Sigma). The mixture was incubated at room temperature for 30 min then mixed in a 1:1 ratio with a 1:1 mixture of the Griess reagent containing 1% sulfanilamide and 0.1% N-(1-naphthyl)-ethylenediamine (NED), both made in a 2.5% H₃PO₄ solution. The entire mixture was incubated for another 5 min prior to reading on a SpectroMax plate reader at absorbance 550nm. Standard curves were generated using NaNO₃ and NaNO₂ to determine the quantity of NO₃⁻ in each sample. To calculate NO₃⁻ consumption, the total amount of NO₂⁻ detected in the absence of *Aspergillus* nitrate reductase was subtracted from the amount of NO₂⁻ in the presence of nitrate reductase, as the Griess reaction specifically detects NO₂⁻. Data were graphed using GraphPad Prism software and analyzed using a plateau followed by one-phase decay non-linear regression model, with the NO₃⁻ curves constrained to a plateau value of 100.

Measurement of membrane potential

An overnight culture of *S. aureus* was diluted 1:200 in 75mL TSB with 100μM NaNO₃ in 125mL flasks to generate a microaerobic environment²¹. Starting at 2.75 hrs (OD₆₆₀ ~0.5), samples were taken every 45 min for measurement of OD₆₆₀, nitrate and acetate levels, and membrane potential, for which approximately 1×10⁶ cfu/mL were added to PBS in a flow cytometry tube. Thirty μM of DiOC₂(3) (3,3'-diethyloxacarbocyanine iodide) dye were added to each tube prior to incubation for 20 min before analysis by flow cytometry. In addition, samples were taken from Bioscreen C plates 16 hrs post-daptomycin treatment and processed as described below. In each experiment, the proton ionophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was used as a control to completely dissipate the membrane potential (Supplementary figure 4b)²⁰. Flow cytometry was performed using an LSRII (BD Biosciences) at the University of Washington Path Flow Facility. Forward and side scatter analysis were used to gate on the population of bacterial cells, and further readings were recorded for 2 × 10⁴ cells with logarithmic signal amplification. Excitation of the dyed cells was accomplished using a 488nm 20mW laser, and fluorescence was collected using filter sets 505LP with 530/30 bandpass for green and 600LP with 610/20 bandpass for red. This protocol allows the generation of a ratiometric profile for changes in ψ that are size-independent. Data were collected using FACS Diva software and analyzed using FlowJo software with the equation: ((Red value - Green value) + 384) generating the derived red/green ratio. Additional statistical analysis was performed with GraphPad Prism software.

Daptomycin sensitivity

Overnight cultures grown in TSB were diluted to OD₆₆₀ ~1.0 before further dilution 1:1000 in TSB with 1mM CaCl₂, with or without the addition of 1mM NaNO₃. Two-hundred seventy µL of the cell suspension were added to each well before incubation of the plate with maximal agitation at 37°C in a BioScreen C plate reader. OD₆₀₀ readings were obtained every 15 min. After 4 hrs of growth, with cells at OD₆₀₀ ~0.4, 30µL of CaCl₂-supplemented TSB ± 100µg/mL of daptomycin were added to bring the total volume to 300µL (10µg/mL daptomycin final concentration). The plates were then placed back into the plate reader and allowed to grow for another 16 hrs. At this point, terminal samples were collected and assessed for membrane potential as described previously. Results were analyzed using GraphPad Prism software and statistical analysis performed using a ratio-paired two-tailed Student's t-test.

Competitive mouse nasal colonisation

Age-matched (6–12 week-old) C57BL/6 and congenic *Nos2^{tm1Lau}/J* (Jackson Laboratory) mice of both sexes were used for these studies. Two weeks prior to infection, the mice began receiving streptomycin (1mg/mL) in their drinking water to eliminate upper respiratory colonisation with commensal *Staphylococcus xylosus*. Streptomycin-resistant derivatives of each *S. aureus* strain were obtained by plating overnight cultures onto TSA plates containing 250µg/mL streptomycin. Isolated streptomycin-resistant colonies were compared to WT strains for colony appearance and growth rate. Overnight cultures of the Str^R WT COL, isogenic *saNOS* mutant and complemented *saNOS* mutant strains were washed in PBS, diluted to 2×10⁸ cfu/mL, then mixed in a 1:1 ratio. Nasal infection was accomplished by anesthetising the mice with isoflurane then administering 20µL of the 1:1 cell mixtures into each nostril. Nasal lavage at subsequent 24–48 hr intervals was performed by anesthetising the mice with isoflurane then lavaging the nasal cavity with 50µL sterile PBS. The recovered nasal lavage fluid was diluted in PBS and plated onto mannitol salt agar plates containing 250µg/mL streptomycin. Colonies were enumerated and patched onto TSA or TSA supplemented with 10µg/mL erythromycin. The competitive indices were determined as the ratio of (mutant/WT)_{out}/(mutant/WT)_{in}. P-values were determined using the Wilcoxon rank-sum test. On the basis of a power analysis, a sample size of n = 10 per group was used to provide 95% confidence that a 1.5-fold or greater difference in competitive fitness could be detected. Mouse infections were performed without randomization or blinding, and were completed with approval of the University of Washington IACUC.

Statistical analysis

Statistics were generated using GraphPad Prism software. Student's t-tests were performed as two-tailed ratio-paired analysis assuming a Gaussian distribution. Non-linear regression of oxygen consumption experiments was completed utilising the one-phase decay model, where y₀ data were constrained to the maximal value, and plateau values were shared. This generated directly comparable rate values, which in addition to values calculated from area under the curve analysis at baseline = 0.01 divided by the total dissolved oxygen, were used in further statistical analysis. Where appropriate data are shown with standard deviation as a measure of statistical variance.

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

Extended Data

Extended Data Figure 3. Effect of *saNOS* on acetate metabolism and NAD^+ and NADH levels
a, c, Growth (dashed lines) and NO_3^- consumption (solid lines) were monitored using the Griess reaction during growth in the presence (or absence; panel **c**) of $100 \mu\text{M}$ NaNO_3 . **b**, Acetate production was monitored to determine TCA cycle flux. The data in panels **a** and **b** represent NO_3^- consumption, growth, and acetate production as measured in the samples used in figure 3 ($n = 4$) $\pm$ s.d. **d**, NAD^+ (solid lines) and NADH (dashed lines) concentrations were determined from WT and *saNOS* strains grown in the presence or absence of supplemental NaNO_3 ($n = 3$) $\pm$ s.d. Data in panel **c** represent the growth and NO_3^- consumption for panel **d**.

Extended Data Figure 4. Maintenance of membrane potential and bacterial growth by saNOS in the presence of daptomycin

a, Mean growth curves of 8 biological replicates of WT and isogenic *saNOS*, *narG* or *saNOS narG* mutants in calcium-supplemented TSB $\pm$ 1 mM NaNO₃ were obtained using a BioScreen C, with (closed symbols) or without (open symbols) the addition of 10 μ g/mL daptomycin after 4 hrs of growth. Plus (+) symbols indicate NO₃ addition. **b**, Mean-derived membrane potential of samples taken from the 20 hr time point during growth in the presence of daptomycin in the Bioscreen C. The protonophore CCCP (Carbonyl Cyanide m-ChloroPhenyl hydrazine) was used as a positive control for ψ collapse in a wild-type strain in the presence of NaNO₃; values below the dotted line are indicative of membrane potential collapse. The data are the means of 3 independent experiments $\pm$ s.d., comprising the last 3 consecutive replicates of the daptomycin growth assay.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Regulation of O_2 respiration by saNOS

a, The rate of O_2 consumption by *Staphylococcus aureus* is enhanced in an saNOS-deficient mutant and diminished in an Hmp-deficient mutant relative to wild-type. Data shown are from a representative experiment ($n = 4$) with lines representing a one-phase decay non-linear regression model. **b**, The rate of O_2 consumption by wild-type cells treated with the NOS inhibitor L-NAME (L- N^G -nitroarginine methyl ester) is enhanced in a dose-dependent fashion. Data shown are from a representative experiment ($n = 4$).

Figure 2. Delayed induction of SrrAB-regulated genes and nitrate respiration in the absence of saNOS

a, Induction of SrrAB-regulated genes was measured as an indicator of menaquinone (MK) pool reduction. Transcript levels were normalised to *ipoD* with graphed values depicting the mean fold-induction $\pm$ s.d. of biological replicates ($n = 4$); a two-tailed ratio-paired Student's t-test was performed with p-values represented as follows: * $P < 0.05$; ** $P < 0.005$. Reduced expression of SrrAB-regulated genes was observed in an saNOS-deficient mutant. **b**, Oxygen consumption (closed symbols; $n = 2$) and nitrate consumption (open symbols; $n = 3$) were simultaneously measured in real-time in cells grown in TSB supplemented with 50 μ M NaNO₃ via a Clark-type electrode and sampling with performance of the Griess reaction, respectively. Shown is the mean of biological replicates $\pm$ s.d. An saNOS-deficient mutant exhibits delayed NarG-dependent nitrate consumption.

Figure 3. Maintenance of membrane potential (ψ) by saNOS

a, Distribution of membrane potential in WT or *saNOS* mutant *S. aureus* as a function of growth phase. Membrane potential is shown as the ratio of red and green fluorescence of the carbocyanine dye DiOC₂(3) and represents the compilation of four independent experiments comprised of 80,000 total cells per time point. An saNOS-deficient mutant is unable to maintain ψ at later time points. **b**, Average mean-derived values for membrane potential $\pm$ s.d. with four replicates per time point. P-values determined by two-tailed ratio-paired Student's t-test are as follows: *P<0.05.

Figure 4. Promotion of daptomycin resistance by saNOS

a, Fold difference in growth after treatment with 10 μg/mL daptomycin in the presence or absence of NO₃⁻. The mean fold-difference ±s.d. in growth between daptomycin-treated and untreated samples at 0, 8, 12, and 16 hrs post-treatment ($n = 8$) is shown. NO₃⁻-dependent enhancement of daptomycin resistance requires saNOS and NarG. **b**, Membrane potential was measured in samples at 16 hrs post-treatment with daptomycin. Data are shown as the mean fold difference ±s.d. in membrane potential in the presence of 1mM NO₃⁻ compared to cells grown without NO₃⁻ ($n = 3$). NO₃⁻-dependent enhancement of daptomycin resistance correlates with the preservation of ψ . P-values determined by two-tailed ratio-paired Student's t-test are as follows: * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$.

Figure 5. Role of *saNOS* in mouse nasal colonisation

The competitive index of *saNOS* mutant *S. aureus* and a wild-type or complemented *saNOS* mutant strain was monitored every 24–72 hrs during nasal colonisation of C57BL/6 or congenic *iNOS*^{-/-} mice ($n = 10$). ø represents mice in which CFU of the *saNOS* mutant were below the level of detection. P-values determined by Wilcoxon rank-sum test are as follows: *P<0.05; **P<0.005; ***P<0.0005.

Figure 6. saNOS maintains $\Delta\psi$ under microaerobic conditions by regulating O_2 and NO_3^- respiration

Under microaerobic conditions $NO\bullet$ derived from L-arginine deimination by saNOS inhibits the terminal aa^3 and bd cytochrome oxidases, diverting electrons to the Nar complex to reduce nitrate to nitrite and maintain the membrane potential ($\Delta\psi$) via a redox loop mechanism. $NO\bullet$ consumption by the Hmp flavohaemoprotein inversely regulates $NO\bullet$ levels in proportion to O_2 availability. (Solid lines represent major reactions, and dashed lines represent minor or potential reactions.)