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Examination of the *Staphylococcus aureus* Nitric Oxide Reductase (saNOR) Reveals its Contribution to Modulating Intracellular NO Levels and Cellular Respiration

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Abstract

Staphylococcus aureus nitrosative stress resistance is due in part to flavohemoprotein (Hmp). Although *hmp* is present in all sequenced *S. aureus* genomes, 37% of analyzed strains also contain *nor*, encoding a predicted quinol-type NO reductase (saNOR). DAF-FM staining of NO-challenged wild-type, *nor*, *hmp*, and *nor hmp* mutant biofilms suggested that Hmp may have a greater contribution to intracellular NO detoxification relative to saNOR. However, saNOR still had a significant impact on intracellular NO levels, and complemented NO detoxification in a *nor hmp* mutant. When grown as NO-challenged static (low-oxygen) cultures, *hmp* and *nor hmp* mutants both experienced a delay in growth initiation, whereas the *nor* mutant's ability to initiate growth was comparable to the wild-type strain. However, saNOR contributed to cell respiration in this assay once growth had resumed, as determined by membrane potential and respiratory activity assays. Expression of *nor* was upregulated during low-oxygen growth and dependent on SrrAB, a two-component system that regulates expression of respiration and nitrosative stress resistance genes. High-level *nor* promoter activity was also detectable in a cell subpopulation near the biofilm substratum. These results suggest that saNOR contributes to NO-dependent respiration during nitrosative stress, possibly conferring an advantage to *nor*⁺ strains *in vivo*.

Keywords

Staphylococcus aureus; nitric oxide reductase; flavohemoprotein; nitrosative stress; DAF-FM; cellular respiration

Introduction

Staphylococcus aureus is an opportunistic pathogen that can cause a diverse array of infections, many of which are due to biofilm formation, such as endocarditis and

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osteomyelitis [reviewed in (Lowy 1998)]. Macrophages and neutrophils of the human immune system release the toxic radical nitric oxide (NO) during the immune cell respiratory burst in response to bacterial infection. NO, along with its reactive nitrogen species (RNS) products (resulting from interaction with reactive oxygen species [ROS]), is known to damage many bacterial cell targets, such as protein iron-sulfur centers, DNA, and lipids [reviewed in (Davis *et al.* 2001)]. NO has also been shown to prevent the insertion of heme groups into proteins, including those found in respiratory proteins such as cytochromes (Waheed *et al.* 2010), which can then be reversed once NO is removed (Waheed *et al.* 2010). *S. aureus* is quite resilient to nitrosative stress (Richardson *et al.* 2006, Richardson *et al.* 2008), in part due to its ability to detoxify NO via a well-characterized flavohemoprotein (Hmp) that is present in all sequenced *S. aureus* genomes to date. The *hmp* gene displays a relatively high level of transcription under low-oxygen conditions compared to aerobic and anaerobic growth (Goncalves *et al.* 2006). Hmp, which oxidizes NO to nitrate (Hausladen *et al.* 2001), has been shown to play a major role in resistance to nitrosative stress *in vitro* and *in vivo* (Richardson *et al.* 2006, Kinkel *et al.* 2013).

Although the role of *S. aureus* Hmp in resistance to nitrosative stress is clear, other enzymatic mechanisms of direct NO detoxification in *S. aureus* have not been explored. In this respect, a previous review article comparing bacterial NO reductase (NOR) enzymes illustrated that the *S. aureus* MRSA252 genome contains a gene (SAR0261) predicted to encode a quinol-type NOR (qNOR) (Hendriks *et al.* 2000). The nucleotide sequence of MRSA252 was also compared to other *S. aureus* strains and *nor* was described as a “genomic islet” because at that time this gene appeared to be unique to MRSA252 (Holden *et al.* 2004). The qNOR-type NO reductases are highly associated with non-denitrifying and/or pathogenic bacteria, and these enzymes differ from the “cytochrome c-type” NOR in that they are encoded by a single gene and they gain electrons directly from quinones during the reduction of NO to N₂O [reviewed in (Hendriks *et al.* 2000)]. In general, NOR enzymes have been shown to be important contributors to both virulence and biofilm formation in human pathogens. For example, accumulation of NO in *nor* mutant biofilms of the denitrifying bacterium *Pseudomonas aeruginosa* caused increased cell death and dispersal (Barraud *et al.* 2006), and the ability of this bacterium to adapt to hypoxic growth in the cystic fibrosis (CF) lung is thought to be an important virulence trait (Worlitzsch *et al.* 2002). Furthermore, the non-denitrifying pathogenic *Neisseria gonorrhoeae* contains a qNOR-encoding gene (*norB*) that was found to be transcriptionally expressed at a higher level under biofilm growth conditions compared to planktonic conditions, and a *norB* mutant had effects on both biofilm attachment and maturation (Falsetta *et al.* 2009). It was subsequently found that NO could induce *N. gonorrhoeae* biofilm growth when nitrite was absent from the medium, and that NO could partially complement the effects of a nitrite reductase mutant (Falsetta *et al.* 2010), suggesting the possibility that NOR-dependent anaerobic respiration occurred in these biofilms. Besides these *in vitro* studies in bacterial biofilms, work in other bacteria have depicted an *in vivo* role for NOR in virulence (Loisel-Meyer *et al.* 2006, Arai *et al.* 2013), intracellular survival in macrophages (Stevanin *et al.* 2005, Loisel-Meyer *et al.* 2006, Kakishima *et al.* 2007, Shimizu *et al.* 2012) and survival in the nasopharyngeal mucosa (Stevanin *et al.* 2005).

To better understand the potential contribution of qNOR to *S. aureus* physiology and virulence, a bioinformatics-based analysis of the prevalence of the *nor* gene in sequenced *S. aureus* strains, as well as characterization of the *nor* gene in the clinical MSSA strain UAMS-1, was undertaken in this study. This investigation revealed that *nor* was present in 37% of the NCBI *S. aureus* genomes analyzed, and these *nor*⁺ strains belonged to sequence types typically associated with healthcare-associated (HA) and livestock-associated (LA) MRSA. A role for saNOR in NO detoxification and anaerobic respiration when grown in static (low-oxygen) NO-challenged cultures was also demonstrated. Expression of *nor* was also found to be dependent on the staphylococcal respiratory response (SrrAB) two-component regulator, that controls expression of anaerobic respiration (Throup *et al.* 2001) and nitrosative stress resistance genes (Richardson *et al.* 2006, Kinkel *et al.* 2013). Based on these results, a role for saNOR in contributing to cellular respiration during nitrosative stress is proposed, which may confer a growth/recovery advantage to the bacterium during infection *in vivo*.

Results

The *nor* gene is highly conserved among a subset of *Staphylococcus aureus* strains

The SAR0261 open reading frame of the sequenced strain MRSA 252 [closely-related to UAMS-1 (Cassat *et al.* 2005)] encodes a 763 amino acid putative qNOR containing 14 predicted transmembrane helices (NCBI Gene Database). The *S. aureus* qNOR amino acid sequence contains domains homologous to the Heme Copper oxidase I superfamily (COG3256, E-value: 1.36e-175), whose members participate in the respiratory chain by reducing O₂ and shunting protons across the membrane, and to the cytochrome C and quinol oxidase polypeptide I domain (Pfam00115, E-value: 3.60e-33). Using ClustalW (Larkin *et al.* 2007), the amino acid sequence of the qNOR from *Geobacillus stearothermophilus* [the organism in which the crystal structure of qNOR was determined (Matsumoto *et al.* 2012)] was aligned with the amino acid sequence of MRSA252 qNOR (**Figure S1**). Not only is the overall amino acid sequence well conserved (98% coverage, 51% identity) between these two proteins, but many amino acids thought to be important for qNOR enzymatic function are also conserved in the putative *S. aureus* qNOR protein. In *G. stearothermophilus*, the crystal-structure predicts that residues His355 and His653 are ligands for heme b, while residue His651 is the ligand for heme b₃ (Matsumoto *et al.* 2012). All three of these histidine residues are conserved in the qNOR of *S. aureus*. In addition, a calcium ion was found to be important for enzyme activity in *G. stearothermophilus* and its ligands Tyr93 and Glu429 (Matsumoto *et al.* 2012) are both conserved in the *S. aureus* qNOR. The residues Glu512 and Glu581 have been implicated in proton transfer during the reduction of NO (Matsumoto *et al.* 2012), and are also present in the qNOR of *S. aureus*. Finally, the residues His508, His559, and His560 that were determined to be important ligands for either Fe_B or Zn_B (Matsumoto *et al.* 2012) are also conserved in *S. aureus* qNOR.

In *S. aureus*, SAR0261 (henceforth referred to as the *nor* gene or saNOR) is located downstream of the *lrgAB* operon (**Figure 1**), which has been associated with the regulation of cell death and eDNA release in *S. aureus* biofilms (Mann *et al.* 2009). Upstream of *lrgAB* is the *scdA* gene, whose expression is highly upregulated in response to nitrosative stress

(Richardson *et al.* 2006, Kinkel *et al.* 2013) and has been implicated in the role of repairing iron sulfur containing proteins in *S. aureus* (Overton *et al.* 2008) and other bacteria (Justino *et al.* 2007). Using the STRING database (Franceschini *et al.* 2013), a close genomic association (whereby two genes are located in close proximity to each other) was found between *scdA* and *nor* homologs in several bacteria, including *Cupriavidus taiwanensis* (formally belonging to the genus *Ralstonia*), *Kangiella koreensis*, *Hahella chejuensis*, *Sinorhizobium meliloti*, *Flavobacterium johnsonia*, *Gramella forsetii*, *Chitinophaga pinensi*, and *Koribacter versatilis*. Although the significance of the *scdA-nor* genomic association in *S. aureus* is not clear, data generated in a *norA* (*scdA* homolog) mutant of *Ralstonia eutropha* suggested that NorA acts as a negative modulator of NorR, an NO-responsive transcriptional regulator that controls expression of the *norA-norB* (qNOR) operon (Busch *et al.* 2005). BLAST searches with the saNOR protein sequence revealed that this qNOR type of NO reductase is also found in several coagulase-negative staphylococci, including *S. simulans* (76% identity), *S. agnetis* (75% identity), and *S. hyicus* (72% identity).

Using the nucleotide sequence for the *nor* gene of MRSA252, a BLAST search was also conducted to assess the presence of *nor* in other NCBI *S. aureus* genomes. Of the 54 *S. aureus* sequenced genomes analyzed (Figure S2), 37% were found to contain the *nor* gene (Table 1). The *nor* nucleotide sequence among these strains was highly conserved, with 100% coverage and 99% identity. Among these *nor*⁺ strains are a number of hospital-associated (HA) strains belonging to clonal complex 30 (CC30) (i.e. MRSA252, n55/2053, TCH60) and sequence-type (ST) 239 (i.e. TW20, JKD6008, BMB9393). As well, a number of livestock-associated (LA) MRSA strains were found to contain *nor*, including ST398, LGA251, 08BA02176, ED133 and 71193 (Table 1). Using the *spa* gene to construct a phylogeny tree of the NCBI *S. aureus* sequenced strains (Figure S2), a divergence was observed, whereby *nor*⁻ strains clustered with “hyper-virulent” or community-acquired strains (i.e. USA300, MW2, COL), while *nor*⁺ strains clustered around “epidemic” HA-MRSA (i.e. MRSA252, ST239 strains, HA-EMRSA-15) and LA-MRSA (i.e. ST398, LGA251) strains. Overall, these data suggest that the previously unstudied *S. aureus nor* gene is common among *S. aureus* strains representing several clinically-relevant genetic lineages.

saNOR and Hmp both regulate NO levels in *S. aureus* biofilm

Since NOR has been shown to effect biofilm structure in other species of bacteria such as *Pseudomonas aeruginosa* (Barraud *et al.* 2006) and *Neisseria gonorrhoeae* (Falsetta *et al.* 2009), and exogenous NO has been shown to decrease biomass in *S. aureus* SA113 (Schlag *et al.* 2007), we initially tested the effects of saNOR and exogenous NO on *S. aureus* static biofilm development. Both 7 hour and 18 hour biofilms of UAMS-1 (wild-type) and isogenic *nor* mutant were grown in the presence and absence of a chemical NO donor and analyzed by confocal microscopy. However, these experiments revealed that mutation of *nor* did not cause any structural changes to the biofilm compared to the wild type strain, regardless of the presence or absence of NO (data not shown). To compare the potential contribution of saNOR and Hmp in regulating intracellular NO levels in *S. aureus* biofilm, UAMS-1 and isogenic *nor* and *hmp* mutants were analyzed using a previously-described DAF-FM diacetate assay (Sapp *et al.* 2014), whereby 7 hour biofilms were harvested,

preloaded with DAF-FM diacetate and then treated with NO donor. These experiments showed that NO-treated *nor* mutant cells displayed a 1.7 fold higher level of DAF-FM fluorescence, whereas the *hmp* mutant had a 3.3 fold higher level of fluorescence compared to the wild type (**Figure 2**). These results were paralleled in the untreated condition, whereby the *nor* and *hmp* mutants each displayed higher levels of DAF-FM fluorescence (suggestive of elevated levels of endogenous NO) compared to wild type (**Figure 2**). Introducing the *nor* and *hmp* genes each in *trans* back into their respective mutants was able to fully and partially complement the DAF-FM fluorescent phenotypes, respectively, under both NO-treated and untreated conditions (**Figure 2**). In order to investigate the possible redundancy of saNOR and Hmp in regulating intracellular NO levels, DAF-FM diacetate staining was subsequently repeated on cells harvested from a *nor hmp* mutant biofilm, as well as double mutant biofilms complemented individually in *trans* with either *nor* or *hmp*. When treated with NO donor, the double mutant had 4.4 fold higher levels of DAF-FM fluorescence compared to the wild type, a phenotype that was able to be partially restored to wild-type levels by both the *nor* and *hmp* complementation plasmids (**Figure 2**). In the absence of exogenous NO, the double mutant also experienced significantly ($p < 0.05$, Dunnett's test) increased endogenous intracellular NO/RNS accumulation compared to the wild-type strain (**Figure 2**). Collectively, these results suggest that although both saNOR and Hmp regulate intracellular NO levels in *S. aureus*, saNOR does not appear to affect static biofilm growth or structure *in vitro*. Furthermore, although Hmp appears to contribute to NO consumption more so than saNOR in the presence of exogenous NO donor, saNOR can compensate for the loss of Hmp when both genes have been mutated.

Expression of *nor* is dependent on growth-phase and oxygen availability

Based on previously-published experimental evidence for NO regulation of biofilm development in *S. aureus* (Schlag *et al.* 2007) and other bacteria (Barraud *et al.* 2006, Falsetta *et al.* 2009), it was surprising that inactivation of *nor* did not lead to altered biofilm structure in UAMS-1. Therefore, to determine if *nor* is expressed during static biofilm growth *in vitro*, we examined biofilm produced by a UAMS-1-derived *nor::gfp* promoter fusion construct using confocal microscopy. Interestingly, these results revealed that high-level *nor* expression occurred in the substratum of the biofilm (**Figure 3A**), presumably where oxygen availability is the lowest. Surprisingly, it also appeared that high-level *nor*-GFP expression only occurred in a subpopulation of cells, as a punctate expression pattern was observed, with only one or a few cells displaying detectable GFP fluorescence in any given location (**Figures 3A-C**). To quantify this expression, flow cytometry was used to calculate the percentage of *nor*-GFP expressing (“*nor*-GFP+”) cells in 7 hour cultures grown as static biofilms and low-oxygen planktonic cultures grown in either TSB or biofilm medium (**Figure 4, Figures S3-S4, and Table 2**). This experiment suggested that *nor* promoter activity was similar across all of the conditions tested, with only about 20% of the total population (“Gate 1”) exhibiting *nor*-GFP fluorescence. Additionally, the scatter plots in **Figure 4** suggest that within this 20% population there is a highly-fluorescent *nor*-GFP expressing sub-population (“Gate 2”) that comprises about 1.3% of the entire cell population. In a parallel analysis, UAMS-1 containing the promoterless GFP plasmid did not yield any detectable fluorescence in Gate 2 (0.01%) while UAMS-1 containing the *cid*-GFP reporter plasmid [previously-reported to be highly active under low-oxygen and static

biofilm growth conditions (Moormeier *et al.* 2013)] yielded a high-level of GFP fluorescence in Gate 2, which comprised the majority of the population (94.2%).

To further characterize *nor* gene expression, quantitative real time PCR was performed on RNA isolated at the early exponential (2 hours), late exponential (6 hours), and stationary (12 hours) phases of growth from wild type UAMS-1 cultures grown both aerobically and under low-oxygen conditions. Relative to the 2 hour aerobic time-point, maximum expression of *nor* occurred during low-oxygen growth, where *nor* RNA levels peaked at late exponential phase, and remained high at early stationary phase (**Figure 5A**). Since *nor* was highly expressed under low-oxygen conditions, its expression was also assessed in two independently generated *srrAB* mutants grown under low-oxygen conditions for 8 hours compared to the UAMS-1 wild type using qRT-PCR. Expression of *nor* in both *srrAB* mutants was decreased by about 40 fold relative to the wild-type strain (**Figure 5B**). Since mutation of *srrAB* is known to confer a modest lag in growth (Throup *et al.* 2001), the promoter activity of *nor* was also monitored over a 24-hour growth period in low-oxygen wild-type and *srrAB* mutant cultures using the plasmid-based *nor*-GFP promoter fusion (data not shown). This analysis revealed that *nor* promoter activity was negligible throughout the monitored growth period, verifying that decreased *nor* RNA levels in the *srrAB* mutant (**Figure 5B**) were not due to delayed growth-phase induction of *nor* expression. Furthermore, *nor* promoter activity was also evaluated in the *srrAB* mutant background using the *nor*-GFP transcriptional reporter plasmid by flow cytometry (**Figure 4 and Table 2**). This analysis revealed that *nor*-GFP fluorescence was detectable in less than 1% of the total cell population, at similar levels to that observed in the promoterless GFP control. Collectively, these results demonstrate that *nor* expression is upregulated during low-oxygen growth in a SrrAB-dependent manner, and high-level *nor* expression appears to only occur in a subpopulation of cells.

saNOR contributes to nitrosative stress resistance by promoting anaerobic respiration

Using the intracellular DAF-FM diacetate stain it was shown that both saNOR and Hmp have a measurable impact on intracellular NO levels, suggesting that saNOR, as previously demonstrated for Hmp (Richardson *et al.* 2006, Kinkel *et al.* 2013), may also contribute to nitrosative stress resistance. The ability of saNOR to mediate recovery from nitrosative stress induced by an NO donor was therefore evaluated by growing planktonic cultures of UAMS-1, *nor*, *hmp*, and *nor hmp* mutants for 24 hours in wells of a static microtiter plate (low-oxygen growth) the presence of 2 mM DPTA/NO. The *hmp* mutant and *nor hmp* double mutant both displayed a delay in recovery, as they both took 8 hours to initiate growth in the presence of DPTA/NO (**Figure 6A**). By comparison, the wild-type and *nor* mutant strains both initiated growth at 5 hours post-inoculation, although the OD₆₀₀ of the *nor* mutant was consistently slightly lower at each measured time point relative to UAMS-1 during the exponential growth phase. These patterns were not observed when UAMS-1 and isogenic mutants were grown in the absence of nitrosative stress (**Figure 6B**). The growth patterns observed in the *nor* mutant under nitrosative stress conditions were complemented by supplying the *nor* gene in *trans* on a plasmid (**Figure S5A-B**). Surprisingly, however, complementation of the *hmp* mutant phenotype was not observed (**Figure S6A-B**), even though the pMK-*hmp* plasmid was able to partially complement the *hmp* mutant phenotype

in the DAF-FM assays presented in **Figure 3**. This is likely due to toxicity associated with overexpressing Hmp, as suggested by a previously-published study (Lewis *et al.* 2008).

The nitrosative stress assays described above suggest that saNOR does not measurably contribute to initial growth recovery in the face of NO challenge. To determine if saNOR plays a more predominant role in resisting NO stress under completely anaerobic conditions (in which Hmp would presumably be inactive due to its requirement for oxygen for NO oxidation), wild-type and isogenic *nor*, *hmp*, and double mutant strains were grown under both low-oxygen and anaerobic conditions in the presence of increasing levels of NO donor (**Figure 7**). This assay revealed that after 12 hours low-oxygen growth, the *hmp* and *nor hmp* double mutant each exhibited significantly ($p < 0.05$) less overall growth relative to wild-type starting at 1 mM exogenous NO donor, whereas the *nor* mutant exhibited growth levels comparable to wild-type at all tested NO concentrations (**Figure 7A**). These results are in line with the 2 mM low-oxygen growth curves presented in Figure 6. When this experiment was conducted under completely anaerobic growth conditions, none of the mutants displayed growth reduction at 12 hours relative to the wild-type strain at any of the tested NO concentrations (**Figure 7B**). However, all of the strains (including the wild-type) did display a 3-fold reduction in overall growth in the presence of 2 mM NO donor compared to untreated wells under the anaerobic growth condition (**Figure 7B**). These results suggest that, under the in vitro conditions and NO levels tested here, Hmp and saNOR do not appear to significantly contribute to anaerobic nitrosative stress resistance. The lack of saNOR contribution to NO resistance during anaerobic growth was not due to decreased *nor* expression, as *nor*-GFP promoter activity was comparable between these anaerobic and low-oxygen growth conditions (**Figure S7**).

Even though the *nor* mutant's growth pattern in the presence of NO appeared to be similar to that of the wild-type strain during exponential growth, the *nor* mutant appeared to plateau at a lower OD₆₀₀ in stationary phase, at about 10 hours growth (**Figure 6A**). This time point correlates with *nor* expression being induced during late exponential/early stationary phase in low-oxygen cultures (**Fig. 5A**), suggesting that saNOR may contribute to low-oxygen growth during late exponential/early stationary phase, possibly by mediating cell respiration using NO as an alternative electron acceptor during nitrosative stress conditions. To test this hypothesis, planktonic cultures of UAMS-1, *nor* mutant, and *nor* complement strains were grown in static microtiter plates for 9 hours (corresponding to the transition from late-exponential to early stationary phase under this growth condition; see **Figure S8**) in both the presence and absence of 2 mM DPTA/NO. Cells from each culture were then harvested, washed with PBS and stained with 4.5 mM 5-Cyano-2,3-ditolyl tetrazolium chloride (CTC), a compound that can be reduced by respiratory dehydrogenases into an insoluble highly-fluorescent CTC formazan product and thus can be used as an indicator of cell respiration. This experiment revealed that the *nor* mutant displayed significantly lower fluorescence and therefore lower respiration compared to wild type when grown in the presence of exogenous NO donor (**Figure 8A**). Furthermore, the *nor* complement strain achieved higher levels of reduced CTC fluorescence compared to the wild-type strain during growth with exogenous NO donor and also during normal growth conditions, suggesting that overexpression of *nor* allowed these cultures to achieve higher levels of respiration (**Figure 8A**). The chemical

DPTA was not available commercially and therefore was not included as a control in this experiment. However, the CTC assay was repeated with the NO donor DETA/NO and DETA alone (DETA is structurally similar to DPTA), and the patterns of CTC fluorescence were comparable to what was observed in the wild-type, *nor* mutant, and complement strains when grown in the presence of DPTA/NO (**Figure S9**). In addition, the DETA alone treatment (the carrier portion of DETA/NO that does not yield NO) did not have any effect on respiration, indicating that the observed changes in CTC fluorescence were caused by NO and not the DPTA or DETA added to the media (**Figure S9**).

In order to determine whether saNOR can function and alter respiration in a strain that does not normally harbor the *nor* gene, *nor* was introduced on a plasmid into LAC-13C, a *nor*-community associated USA300 MRSA strain. LAC-13C with and without empty vector, as well as LAC-13C transformed with the *nor* plasmid (LAC-*nor*+), were grown in the presence and absence of DPTA/NO donor for 9 hours, and subjected to the CTC assay described above. It was observed in these experiments that the LAC-*nor*+ strain had significantly higher levels of CTC fluorescence compared to its parent strain with or without the empty vector, in both the untreated and NO treatment conditions (**Figure 8B**). It was also shown that LAC-*nor*+ had a higher level of respiration under the NO conditions than untreated conditions (**Figure 8B**). To further confirm a role for saNOR in promoting respiration during nitrosative stress, 9 hour low-oxygen cultures of UAMS-1 (wild-type), *nor* mutant, and *nor* complement strains were grown in the presence or absence of 2 mM DPTA/NO and stained with the membrane potential stain 3,3'-diethyloxacarbocyanine iodide [DiOC₂(3)]. This carbocyanine dye initially stains all cells (fluorescing green), followed by aggregation of the stain in a membrane potential (Ψ)-dependent manner, which will cause a fluorescence shift from green to red. Using flow cytometry to measure the ratio of red to green fluorescence of each cell accounts for differences in cell size and degree of clumping (Shapiro *et al.* 2004). In the untreated condition there was no change in Ψ (red: green ratio) observed among any of the strains tested. However, when grown in the presence of NO donor, a positive shift in the red: green ratio (indicating an increased Ψ) was observed in the UAMS-1 and *nor* complement strains compared to each of their respective untreated conditions, whereas the *nor* mutant strain did not undergo this positive shift when grown in the presence of NO donor (**Figure 9**). These results provide further evidence to support the hypothesis that saNOR plays a metabolic role in *S. aureus* by contributing to cellular respiration during growth under low-oxygen nitrosative stress conditions.

Discussion

The data presented in this study suggest that saNOR makes two possible contributions to *S. aureus* physiology under nitrosative stress conditions: 1) Direct detoxification of NO (by reducing NO to N₂O), which would also protect the cellular respiration machinery from NO-induced damage, and 2) Contribution to anaerobic respiration by using NO as an alternative electron acceptor. By fulfilling this latter role, the enzymatic activity of saNOR may also aid in maintaining redox balance in the respiratory quinone pool. NO is known to interfere with the incorporation of heme groups in many respiratory proteins causing respiration to stall (Waheed *et al.* 2010) and also competes with oxygen at terminal oxidases (Brown *et al.*

1997), both events that can cause electrons to accumulate within the quinone pool. In fact, this reduced electron flow has been proposed to be the signal sensed by the *S. aureus* SrrAB two-component system, which responds by activating expression of various nitrosative stress resistance and metabolic genes (Kinkel *et al.* 2013). Since saNOR is a quinol-type NO reductase, it is predicted to receive its electrons directly from reduced quinones in the electron transport chain when catalyzing NO reduction (Matsumoto *et al.* 2012). Since *S. aureus* only uses menaquinone in the respiratory chain (Kohler *et al.* 2008, Kinkel *et al.* 2013), saNOR could potentially contribute to metabolic adaptation to nitrosative stress by both directly oxidizing the quinone pool (and thereby allowing increased flow of electrons through the respiratory chain) and by utilizing NO as an alternative electron acceptor. Although NO is not as energetically favorable as other alternative electron acceptors (i.e. oxygen, nitrate, nitrite), under nitrosative stress conditions when much of the respiratory machinery may be damaged, the use of NO as the terminal electron acceptor could help maintain membrane potential as well as the ratio of reduced to oxidized quinone levels. Further experimentation will be required to determine if saNOR contributes to cell physiology in this exact manner.

The involvement of saNOR in metabolic adaptation to nitrosative stress is also supported by the observation that *nor* expression is strongly dependent on the SrrAB two-component system (**Figures 4-5**). In fact, analysis of the DNA sequence directly upstream of *nor* using Virtual Footprint software (Munch *et al.* 2005) revealed the presence of a putative SrrA (ResD) binding site (data not shown), suggesting a direct interaction of SrrA with the *nor* promoter region. Previous microarray analyses of genes upregulated in response to nitrosative stress have identified a number of metabolic and NO resistance genes, including *hmp*, *ldh1*, and *qox* (Richardson *et al.* 2006, Kinkel *et al.* 2013). However, these studies overlooked the potential role of saNOR in contributing to the nitrosative stress response because they were conducted in *S. aureus* strain COL, which does not contain *nor* in its genome. Although *nor* expression was up-regulated during low-oxygen growth relative to aerobic growth (**Figure 5**), *nor* expression was not found to be induced by the addition of NO during low-oxygen growth (data not shown). Additionally, *nor* promoter activity appeared to be highly active in the substratum of static biofilms (**Figure 3**), an area presumed to be at lower oxygen concentrations relative to the biofilm surface. Collectively, these data indicate that saNOR is highly expressed in the absence of exogenous nitrosative stress and therefore may also be involved in endogenous NO metabolism. For example, under low-oxygen growth conditions there may be endogenous sources of NO production that saNOR can act upon to promote anaerobic respiration, such as bacterial nitric oxide synthase (saNOS). In this respect, expression of the *nos* gene was also recently shown to be upregulated in *S. aureus* during low-oxygen growth (Sapp *et al.* 2014). Another potential source of endogenous NO in *S. aureus* when grown under low-oxygen conditions could be nitrite produced from the reduction of nitrate [Reviewed in (Moreno-Vivian *et al.* 1999)], which under low pH conditions could spontaneously undergo disproportionation to yield endogenous NO.

The significance of the punctate pattern of high-level *nor*-GFP expression observed in the biofilm substratum (**Figure 3**) has yet to be ascertained. One possibility is that saNOR needs

to be present in the membrane before the cells are subjected to exogenous nitrosative stress, in order to quickly counteract the deleterious effects of NO stress on cellular respiration. In this scenario, high-level synthesis of saNOR by every member of the cell population may be energetically inefficient. Alternatively, saNOR expressing cells may be able to detoxify NO for their neighboring cells. This punctate expression could also be due to microenvironments where there are pockets of low-oxygen areas coinciding with a secondary signal. A discrepancy appears to exist in the *nor*-GFP expression results described in Figures 3 and 4, in that the confocal microscopy images depict a smaller *nor*-GFP expressing sub-population than indicated by “Gate 1” of the flow cytometry results. This could be due to the fact that the confocal microscope may have underestimated the number of GFP+ cells in the biofilm if some of the *nor*-GFP+ cells were washed away during processing and staining of the biofilm. Alternatively, it is possible that the highly-fluorescent *nor*-GFP+ subpopulation (“Gate 2”, **Figure 4**) actually coincides with the punctate high level *nor*-GFP fluorescence observed by confocal microscopy. Bi-stability of gene expression is a hallmark of phenotypic “bet-hedging”, a strategy employed by bacteria to ensure survival of a subpopulation under adverse stress conditions or nutrient limitation (Kussell *et al.* 2005). Although variation in gene expression has been suggested to contribute to the generation of metabolically-diverse subpopulations within a bacterial community, little is known about what role, if any, stochastic control plays in this process (Lidstrom *et al.* 2010). Plasmid-based fluorescent reporters are routinely used to study *S. aureus* promoter activity (Cheung *et al.* 1998, Yarwood *et al.* 2004, Malone *et al.* 2009, Moormeier *et al.* 2013), and pMK4 specifically has been used for both GFP and *lux* transcriptional reporter studies (Francis *et al.* 2000, Shompole *et al.* 2003). This being said, the subpopulation behavior of *nor* promoter activity observed in our plasmid-based reporter system will need to be verified using a single-copy chromosomal integration plasmid (Luong *et al.* 2007) prior to pursuing future investigations into the potential stochastic regulation of *nor* promoter activity. However, the fact that punctate expression of *nor*-GFP in the biofilm seemed to occur in only one or a few distinct cells in any given field of view is suggestive of stochastic regulation of expression, as has been observed with the regulation of the production of extracellular matrix in *Bacillus subtilis* biofilms (Chai *et al.* 2008), production of chitinase in the marine bacterium *Pseudoalteromonas* species (Baty *et al.* 2000), and differential expression of nuclease expression during *S. aureus* biofilm development (Moormeier *et al.* 2014).

Our bioinformatics analysis revealed that many *nor*+ *S. aureus* isolates are associated with LA-MRSA and HA-MRSA strains belonging to the “epidemic” MRSA groups 15 and 16, while strains such as COL, Newman, and USA300/400 CA-MRSA, that are thought of as being more virulent, lack *nor* in their genomes. Our studies have also shown that saNOR is functional in LAC-13C, a strain that normally lacks the *nor* gene (**Figure 8B**), suggesting that the ability to integrate this enzyme's function in cellular respiration is not unique to the genomic *nor*+ strains. It is unclear from our bioinformatics analysis whether *nor*+ strains gained this gene via horizontal gene transfer, or if *nor*- strains lost the gene at some point in their micro-evolution. saNOR may allow *S. aureus* strains to survive longer on common colonization sites such as the skin and nares, thereby increasing the chances of transmission. For example, saNOR could help combat the nitrosative stress at these sites as gaseous NO is

exhaled from humans (Gustafsson *et al.* 1991) and high concentrations of NO have been found from nasal exhalation within the nasopharynx (Kimberly *et al.* 1996, Andersson *et al.* 2002). Likewise, colonization on skin has been shown to be hampered by human skin cells producing NO via nitric oxide synthases [Reviewed in (Cals-Grierson *et al.* 2004)]. As *nor* is highly expressed under low-oxygen conditions, it is also plausible that *nor*⁺ strains may have a competitive advantage in low-oxygen infection environments such as abscesses (Park *et al.* 1992), or alternatively, when exposed to high-levels of NO, such as when engulfed by immune cells undergoing respiratory burst (Wright *et al.* 1989, Panaro *et al.* 2003). It will be exciting to determine the exact relationship between *nor* and the colonization potential, transmissibility, and/or virulence of LA-MRSA and HA-MRSA strains.

Material and Methods

Bacterial strains and growth conditions

Strains of *S. aureus* and *Escherichia coli* used in this study are listed in **Table S1**. All *E. coli* strains were cultured on Luria-Bertani (LB) agar or LB broth, containing selective antibiotic as appropriate. Unless otherwise indicated, all *S. aureus* cultures were grown at 37°C (ambient atmosphere), in either tryptic soy broth (TSB) or TSB containing a total of 0.75% (wt/vol) glucose and 3.5% (wt/vol) NaCl (biofilm medium) as indicated for each experiment. For all static biofilm experiments, plate wells were coated with 20% (vol vol⁻¹) human plasma in bicarbonate buffer (Sigma) as previously-described (Rice *et al.* 2007) prior to culture inoculation. Before beginning each experiment, *S. aureus* was freshly streaked from -80°C glycerol stocks onto tryptic soy agar (TSA) containing selective antibiotic, as required (**Table S1**), for 24 hours. Antibiotics were used at the following concentrations: Ampicillin (Amp) 50 µg ml⁻¹, Kanamycin (Km) 50 µg ml⁻¹, Spectinomycin (Spec) 100 µg ml⁻¹, Erythromycin (Erm) 2 or 10 µg ml⁻¹, Tetracycline (Tet) 5 µg ml⁻¹, Chloramphenicol (Cm) 1, 5, or 10 µg ml⁻¹. Stocks of all strains were stored at -80°C in a 1:1 ratio of overnight culture to 50% glycerol (vol vol⁻¹) in cryogenic tubes.

Creation of *nor*, *hmp*, and *srrAB* mutant strains

Disruption of the *nor* gene in the UAMS-1 background was accomplished by the insertion of the unmarked group II intron encoded within the TargeTron system (Sigma-Aldrich) as previously described (Yao *et al.* 2006). The MRSA252 genome SAR0261 (*nor*) gene sequence was analyzed by the TargeTron algorithm and possible insertion sites and primers were designed. Primers IBS, EBS1d, and EBS2 were used per manufacturer's directions to redirect the intron insertion site, in the plasmid, pNL9164 (Sigma Aldrich) to the location 390 bp downstream of the *nor* ORF translation start site. Following sequencing of the plasmid, it was then electroporated into RN4220 and then into the UAMS-1 background where integration was achieved by the addition of cadmium chloride and selection on Erm as previously described (Yao *et al.* 2006). Possible mutants were screened for insertion by PCR with primers flanking the *nor* gene (nor4-F and nor4-R). The resulting isolate was named KB1031.

To create *hmp* and *hmp nor* mutants, 5' and 3' flanking regions of the *hmp* gene were PCR amplified with primer pairs hmp1-F/R and hmp2-F/R, respectively, using Thermalace DNA

polymerase (Invitrogen Life Technologies) and UAMS-1 genomic DNA as template. Each PCR product was cloned into pCRBlunt (Invitrogen Life Technologies) following the manufacturer's protocols for ligation and *E. coli* transformation. Each PCR product was then excised from pCRBlunt by restriction digestion, followed by gel-purification of each insert (DNA Gel Extraction Kit, Zymo Research) and sequential cloning into the shuttle vector pBT2 (Bruckner 1997). The resulting pBT2- *hmp* plasmid was then confirmed by restriction digestion and agarose gel electrophoresis, followed by electroporation into RN4220 and phage-transduction into both UAMS-1 (wild type) and KB1031 (*nor* mutant), with growth at 30°C for all steps (Shafer *et al.* 1979, Schenk *et al.* 1992, Groicher *et al.* 2000). Temperature-sensitive homologous recombination of pBT2- *hmp* into each strain and subsequent selection for a second recombination event were performed as previously-described (Sapp *et al.* 2014), resulting in a 1037 bp deletion of the *hmp* gene in both UAMS-1 and in the *nor* mutant. Mutants containing the correct *hmp* deletion allele were then confirmed by PCR and agarose gel electrophoresis.

To create strain KCR10 (*srrAB* allele-replacement mutant; *Erm*^R), PCR products corresponding to the 5' and 3' regions spanning the *srrAB* locus were amplified with primer pairs *srrA1-F/srrA1-R* and *srrB1-F/srrB1-R*. Each PCR product was cloned into pCRBlunt as described above. The *srrA1* and *srrB1* inserts were each excised from pCRBlunt by restriction digestion, gel-purified, and sequentially ligated into cloning sites located upstream and downstream of the *Erm* resistance gene in pDG647 (Guerout-Fleury *et al.* 1995). The resulting vector was then digested to liberate the 5'*srrA*-*Erm*-3'*srrB* fragment, which was gel-purified and ligated to pCL52.2 (Sau *et al.* 1997) to create pCL- *srrAB*. This plasmid was transformed into strain RN4220 by electroporation, followed by phage transduction into UAMS-1, with growth at 30°C for all steps (Shafer *et al.* 1979, Schenk *et al.* 1992, Groicher *et al.* 2000). Temperature-sensitive homologous recombination of pCL- *srrAB* into the UAMS-1 chromosome and subsequent selection for a second recombination event were performed as previously-described (Sapp *et al.* 2014). PCR and Southern blotting were used to screen candidate mutants to confirm that 1586 bp of the *srrAB* operon had been correctly replaced by the *erm* resistance gene.

To create strain KB6004 (unmarked *srrAB* deletion mutant), PCR was performed with primers IW91c and IW92c and UAMS-1 template DNA to amplify the 5' region upstream of *srrAB*. This PCR product was then restriction digested and cloned into pCL52.2 (Sau *et al.* 1997). A second PCR to amplify the 3' region downstream of *srrAB* was performed with primers IW93c and IW94c, followed by restriction digestion and cloning into pCL52.2 containing the IW91c-IW92c PCR insert, to produce plasmid pIHW45. This plasmid was moved into RN4220 and then into UAMS-1 using electroporation and phage transduction, respectively. Temperature-sensitive homologous recombination of pIHW45 into the UAMS-1 chromosome and subsequent selection for a second recombination event were performed as previously-described (Sapp *et al.* 2014), except that a 45°C non-permissive temperature was used. The mutation was confirmed by PCR.

Cloning of *nor* and *hmp* complementation plasmids

To create a *nor* complement plasmid, a 2913 bp PCR product spanning the entire *nor* ORF and upstream putative promoter region was amplified by PCR with primers nor3-F/ nor3-R (**Table S2**) using AccuPrime™ Pfx SuperMix proofreading polymerase (Invitrogen Life Technologies) and UAMS-1 genomic DNA as template. The PCR product was first cloned into pCRBlunt, then excised by restriction digestion and cloned into the shuttle vector pMK4 (Sullivan *et al.* 1984). The resulting pMK*nor* plasmid was then moved into *S. aureus* RN4220 by electroporation, then into the UAMS-1 *nor* mutant, *hmp nor* double mutant, and strain LAC-13C via Φ 11-mediated phage transduction (Shafer *et al.* 1979, Schenk *et al.* 1992, Groicher *et al.* 2000). For *hmp* complementation, a 2249 bp PCR product (spanning the *hmp* ORF and its upstream promoter region) was amplified using primers hmp3-F/R and cloned into pCRBlunt as described above. The *hmp* PCR product was then excised by restriction digestion and cloned into pMK4. Once confirmed by restriction enzyme digestion, the plasmid was then moved into *S. aureus* RN4220 by electroporation and into both the UAMS-1 *hmp* mutant strain and the UAMS-1 *hmp nor* double mutant via phage transduction as described above. To create vector-only control strains, plasmid pMK4 was also phage-transduced into UAMS-1, UAMS-1 *nor* mutant, *hmp* mutant, *nor hmp* mutant, and LAC-13C.

Creation of *nor*-GFP, *cid*-GFP, and promoterless GFP transcriptional reporter plasmids

The 600 bp sequence upstream of the *nor* ATG start codon was PCR amplified with primers nor2-F/nor2-R (**Table S2**) using UAMS-1 genomic DNA as template. GFPmut3 (NCBI gene accession #: DQ493885.2) was also amplified using primers GFP-F/GFP-R (**Table S2**) and template pCR-GFPmut3, which contains a 28-bp translation enhancer region upstream of the GFPmut3 ORF (Miller *et al.* 1997). PCR products were each cloned into pCRBlunt as described above, then liberated from pCRBlunt by restriction enzyme digestion and sequentially cloned into pBT2 (Bruckner 1997). The resulting *nor*-GFP fragment was then cut out of pBT2 and cloned into pMK4 (Sullivan *et al.* 1984), and transformed into *E. coli*. The confirmed construct was electroporated into *S. aureus* RN4220 and then phage-transduced into *S. aureus* UAMS-1 and KCR10 (*srrAB* mutant) as described above. A promoterless pMKGFP plasmid (negative control) was created by excising the *nor* promoter from pMK*nor*GFP, followed by treatment with DNA polymerase I Klenow (New England Biolabs), ligation and transformed into *E. coli* as described above. Loss of the *nor* promoter in this construct was confirmed by restriction digestion and gel electrophoresis, followed by movement into *S. aureus* RN4220 by electroporation and subsequent phage transduction into UAMS-1 and KCR10. A positive control was created by PCR amplification of the promoter region of the *cidA* gene with primers cidA-F/R, followed by cloning into pCRBlunt as described above. The *cidA* promoter was then excised from pCRBlunt by restriction digestion, and ligated into gel purified linear pMKGFP. The resulting pMK-*cid*GFP construct was then electroporated into *S. aureus* RN4220 and moved to *S. aureus* UAMS-1 by phage transduction (Shafer *et al.* 1979, Schenk *et al.* 1992, Groicher *et al.* 2000).

Bioinformatics

NCBI nucleotide BLAST (Altschul *et al.* 1990) was used to align the nucleotide sequence of the *nor* gene (SAR0261) of *S. aureus* strain MRSA252 to other *S. aureus* strains with complete genome sequences. NCBI protein BLAST (Altschul *et al.* 1990) was used to align the predicted amino acid sequence of the MRSA252 *nor* gene to the amino acid sequence of *S. aureus* strains with complete genome sequences as well as other bacteria. ClustalW (Larkin *et al.* 2007) was used to align the predicted amino acid sequence of saNOR (MRSA252) to the amino acid sequence of qNOR from *G. stearothermophilus*. The STRING database was used to investigate associations between *nor* and other genes (Snel *et al.* 2000). A cladogram was constructed using the *spa* gene sequence from all available complete *S. aureus* genomes through NCBI and the “one click” mode in the phylogeny.fr online program (Dereeper *et al.* 2008). “One click” uses MUSCLE for alignment, Gblocks for curation, PhyML for phylogeny, and TreeDyn for tree rendering (Castresana 2000, Guindon *et al.* 2003, Edgar 2004, Anisimova *et al.* 2006, Chevenet *et al.* 2006, Dereeper *et al.* 2008, Dereeper *et al.* 2010).

RNA isolation and analysis of gene expression by quantitative real-time PCR (qRT-PCR)

To compare *nor* expression under low-oxygen and aerobic growth conditions, overnight culture of UAMS-1 was diluted in TSB to an OD₆₀₀=0.05, and grown as either low-oxygen planktonic (7:10 volume to flask ratio, 0 RPM) or aerobic (1:10 volume to flask ratio, 250 RPM) cultures (n=3 biological replicates per growth condition). Cells were harvested at 2 hour (early exponential phase), 6 hour (late exponential phase), and 12 hour (early stationary phase) time points post-inoculation, followed immediately by RNA isolation. To investigate the effect of SrrAB on *nor* expression, overnight cultures of wild type and each *srrAB* mutant were diluted to an OD₆₀₀=0.05 in TSB and grown under low-oxygen conditions as described above. Cells were harvested from 3 biological replicates of each culture after 8 hours growth, followed immediately by RNA isolation. In all experiments, RNA was isolated following published methods (Patton *et al.* 2005, Sapp *et al.* 2014). DNase-treatment of RNA samples, cDNA synthesis, and qRT-PCR were performed as previously-described (Sapp *et al.* 2014). The *nor1-F/R* primers were used to detect expression of *nor*, and *sigA* was the reference gene for all experiments (Table S2). All gene expression changes were calculated using the Livak method (2^{-C_t}) (Livak *et al.* 2001).

Biofilm DAF-FM diacetate assays

A previously published protocol (Sapp *et al.* 2014) was followed to measure relative intracellular NO levels using DAF-FM diacetate staining. In brief, overnight cultures were diluted to an OD₆₀₀=0.05 in fresh biofilm medium, and 1 ml aliquots were used to inoculate wells of a 24 well plate (Costar 3524). Static biofilms were grown for 7 hours, followed by harvesting of the whole well (biofilm + planktonic cells) and resuspension in 1x Hank's Buffered Salt Solution (HBSS) containing 5μM 4-Amino-5-Methylamino-2',7'-Difluorofluorescein (DAF-FM diacetate, Invitrogen Life Technologies). Cell suspensions were incubated at 37°C for 1 hour, followed by centrifugation and washing with 1 mL of HBSS. Washed cells were then either resuspended in 650μL of HBSS alone or in HBSS containing 100 μM DEA/NO (chemical NO donor, half-life = 2 min at 37°C, Cayman

Chemicals). Samples were immediately loaded into a 96 well plate (Costar 3904) and the fluorescence (EX/EM 485±10/516±10) (RFU) and OD₆₀₀ readings of each sample were measured with a Biotek Synergy HT fluorescent microplate reader. Fluorescence (RFU/OD₆₀₀) was reported as relative fluorescence units (RFU) measured at 30 min divided by the OD₆₀₀ reading of each well. Relative fold-change in DAF-FM fluorescence for each experiment was calculated by dividing each RFU/OD₆₀₀ value by the average RFU/OD₆₀₀ of the wild-type strain.

Measurement of nor-GFP fluorescence by flow cytometry

Overnight cultures of UAMS-1 pMK_{nor}GFP, UAMS-1 pMK_{cid}GFP, UAMS-1 pMKGFP, KCR10 pMK_{nor}GFP, and KCR10 pMKGFP grown in either TSB or biofilm medium were diluted to an OD₆₀₀=0.05 and grown for 7 hours under low-oxygen planktonic conditions as described above. For static biofilm growth, overnight cultures were each diluted into 9 mL biofilm medium to an OD₆₀₀=0.05 and grown in wells of a 6-well microtiter plate (Costar 3516) for 7 hours. Cells from each growth condition were then harvested, washed once in HBSS and then diluted for flow cytometry. Samples were analyzed on a FACSsort flow cytometer (BD-Biosciences, San Jose, CA), which incorporates a 15 milliwatt laser emitting at 488 nanometers (nm). GFP fluorescence was collected using a 530 ± 15 nm bandpass filter. A threshold was adjusted on log-side scatter while buffer was analyzed to reject background particulates and instrument noise. Data for 50,000 cells were collected per sample. The “Gate 1” and “Gate 2” regions for counting fluorescent cells was defined on a GFP-fluorescence vs side light scatter plot, based on negative control (UAMS-1 pMKGFP) samples. Cell Quest version 3.3 software was used for graphics and calculation of percent-positive fluorescent cells.

Confocal microscopy

UAMS-1 pMK_{nor}GFP, UAMS-1 pMKGFP, and UAMS-1 *cid*AGFP static biofilms were grown for 7 hours at 37°C (ambient atmosphere) in 96 well optically clear plates (costar 3720). Biofilms were then stained with 1 mM Syto83 nucleic acid stain (Invitrogen) for 30 min and 37°C. After this incubation period, the stain was carefully removed and replaced with 0.85% NaCl. Biofilms were then imaged at 400x magnification (Zeiss 40x/1.2W) with a Zeiss LSM Pascal scanning confocal microscope (EX: 488 nm, EM: BP 505-530 nm [green fluorescence], LP 560 nm [red fluorescence]).

Low-oxygen Planktonic Growth Curves

To monitor the growth of cultures in the presence or absence of nitrosative stress, overnight cultures were each diluted to an OD₆₀₀=0.05 in TSB with or without the presence of 2 mM DPTA/NO (chemical NO donor, half-life = 3 hr at 37°C, Cayman Chemicals) at the time of inoculation. Triplicate wells on a 96 well plate (costar 3904) were then loaded with 200 µL of inoculum each and grown statically for 24 hours at 37°C (ambient atmosphere). Every hour the plate was shaken for 5 seconds at medium speed and the OD₆₀₀ of the wells were measured with a Biotek Synergy HT fluorescent microplate reader.

Anaerobic nitrosative stress assays

To monitor the growth of cultures in the presence or absence of nitrosative stress under anaerobic conditions, overnight cultures were each diluted to an $OD_{600}=0.05$ in 1 ml TSB and transferred to wells of a 24-well plate (Costar 3524). These wells were grown in the absence or presence of DPTA/NO (0.25 mM, 0.5 mM, 1.0 mM, or 2.0 mM final concentration) at the time of inoculation for 12 hours at 37°C in an Anoxomat anaerobic system (Mart Microbiology, The Netherlands), prior to reading the OD_{600} of each well with a Biotek Synergy HT microplate reader. For comparison, a duplicate plate was grown under low-oxygen conditions as described above.

Measuring Respiration with CTC staining

To measure differences in respiration between the UAMS-1 wild type, *nor* mutant, and *nor* complement, as well as strain LAC-13c, LAC-13c pMK4, and LAC-13c pMK*nor*, overnight cultures of each strain were diluted to an $OD_{600}=0.05$ in TSB, and 1 ml aliquots were used to inoculate wells of a 24 well plate (Costar 3524). Cultures were either left untreated or treated with 2 mM DPTA/NO at time of inoculation. After 9 hours static growth (37°C, ambient atmosphere), cells were then harvested, washed with Phosphate-buffered saline (PBS) and stained with 4.5 mM CTC. Samples were immediately loaded into a 96 well plate (costar 3904) and the fluorescence (RFU) (EX/EM 485±20nm/645±40nm) and OD_{600} readings of each sample were measured with a Biotek Synergy HT fluorescent microplate reader. Fluorescence reads at 70 min were normalized by dividing the RFU of each well by its corresponding initial OD_{600} reading. To monitor growth in a parallel experiment, cultures were treated and grown in a 24 well plate (Costar 3524) according to the procedure for CTC staining described above. OD_{600} reads were taken at time of initial inoculation, 3 hr, 6 hr, 9 hr, 12 hr, and 24 hr growth using a microplate reader.

Membrane Potential Staining

Low-oxygen planktonic TSB cultures were grown in 24 well microtiter plates for 9 hours at 37°C as described above. The entire well of culture for each sample was harvested and washed with PBS. Cells were then stained using the BacLight™ Bacterial Membrane Potential Kit (Invitrogen) as previously described (Novo *et al.* 1999, Patton *et al.* 2006). Briefly, samples were diluted in 1mL of PBS and 20µL of dilution was added to 2 mL of 30 µM of the membrane potential stain 3,3'-diethyloxycarbocyanine iodide (DiOC₂(3)). A negative control was prepared by adding the depolarizing agent carbonyl cyanide 3-chlorophenylhydrazone (CCCP) during the staining step to a concentration of 5 µM. Samples were analyzed on an LSR-II flow cytometer running Diva 6.1.3 software (BD-Biosciences, San Jose, CA), which uses a 100 milliwatt laser emitting at 488 nanometers (nm) for light scatter detection and fluorescence excitation. Forward (FSC) versus side light scatter (SSC) were displayed on a log-log plot. A threshold of SSC = 1000 was set to exclude most background noise based on the buffer-only and stain-only samples. A gate was placed surrounding the major peak, which was just above the SSC threshold. Green fluorescence was collected at 530 ± 15 nm and red fluorescence was collected at 610 ± 10 nm. A ratio parameter was created as red fluorescence/green fluorescence. Using the CCCP-depolarized control sample, the ratio was scaled to the left side of a histogram (scale factor

of 6.00). All further samples were analyzed using these settings. Fifty-thousand particles were collected in a data file and the median value of the red/green fluorescence ratio was computed for each sample.

Statistical Analyses

All statistical analyses were performed using Sigmaplot version 12.5 (Build 12.5.0.38, Systat Software, Inc.). Data was tested for normality and equal variance, followed by a parametric (if normality and/or equal variance tests passed) or non-parametric (if normality and/or equal variance tests failed) one-way ANOVA and appropriate multiple-comparison test to detect differences between individual groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Genomic context of *nor* in *S. aureus* MRSA252

The predicted function of each ORF is indicated under its respective gene, and promoter region of each gene is indicated by arrows.

Figure 2. Comparison of the contribution of saNOR and Hmp to intracellular NO levels
 Static biofilms of each indicated strain were harvested after 7 hours growth, preloaded with 5 μ M DAF-FM diacetate for 1 hour at 37°C, and then either treated with 100 μ M DEA/NO in HBSS buffer (dark gray bars) or HBSS alone (untreated, light gray bars). Fluorescence (RFU) and OD₆₀₀ readings of each sample were then measured with a fluorescent microplate reader. Relative fold-changes in DAF-FM fluorescence for all experiments were calculated by dividing the RFU/OD₆₀₀ measurement obtained for each strain by the average RFU/OD₆₀₀ of the wild-type strain. Data represent the average of n=4 independent experiments $\pm$ standard error of the mean (SEM). *denotes statistically significant difference compared to untreated UAMS-1 (wild-type strain), •denotes statistically significant difference compared to NO-treated UAMS-1 (Dunnett's test, p<0.05).

Figure 3. *nor*-GFP expression within UAMS-1 static biofilm

UAMS-1 pMK*nor*GFP biofilms were grown in optically-clear 96-well plates for 7 hours, followed by staining with Syto83 (red fluorescence) and confocal microscopy to visualize the entire biofilm. High-level *nor*-GFP fluorescence (green) was observed in the bottom layers of the biofilm, as shown by orthogonal images with the dark blue line set at 5 μ m (A), 11 μ m (B), and 15 μ m (C) from the bottom of the z-stack (400x magnification). Images are representative of 12 fields of view acquired in 3 independent experiments.

Figure 4. *nor*-GFP fluorescence in static biofilm cultures quantified by flow cytometry UAMS-1 pMKnorGFP, *srrAB* mutant pMKnorGFP, UAMS-1 pMKGFP (negative control, no promoter), and UAMS-1 pMKcidGFP (positive control, highly-active *cidA* promoter) 7 hour biofilms were harvested (biofilm + supernatant), resuspended in HBSS, and subjected to flow cytometry to quantify the percentage of GFP+ cells (Gate 1). Representative scatter plots are shown, whereby GFP fluorescence (X axis) is plotted as a function of side scatter (SSC; Y axis). Gate 1 was defined based on negative control (UAMS-1 pMKGFP) samples, and Gate 2 was defined based on the highly-fluorescent *nor*-GFP subpopulation. The average %GFP+ cells for each gate ± SEM (n=3 biological replicates acquired over 2 independent experiments) is indicated underneath each graph.

Figure 5. qRT-PCR analysis of *nor* expression

A: RNA was isolated from UAMS-1 grown under planktonic low-oxygen (light grey bars) and aerobic (dark grey bars) conditions at 2, 6, and 12 hour time points. The RNA samples were converted to cDNA and qRT-PCR was performed using primers for *nor* and *sigA* (reference gene). The 2 hour aerobic condition was used as the calibrator sample, and relative fold-change expression was calculated using the Livak method. *Denotes significant difference relative to 2 hour aerobic (Tukey Test, $p < 0.05$). **Denotes significant difference relative to 2 hour low-oxygen (Tukey test, $p < 0.05$). Although not noted on the graph, there is also a significant difference between aerobic and low-oxygen conditions at 6 hour (t-test, $p = 0.013$) and 12 hour growth (t-test, $p < 0.001$). **B:** RNA was isolated from 8 hour cultures of UAMS-1 and two independently-made *srrAB* mutants grown under planktonic low-oxygen conditions. The RNA samples were converted to cDNA and qRT-PCR was performed as described above. The UAMS-1 wild type sample was used as the calibrator, and fold change was calculated using the Livak method. *Denotes significant difference relative to UAMS-1 wild type (Dunnett's Method, $p < 0.05$). For both graphs, qRT-PCR data represent the average $\pm$ SEM of $n = 3$ biological replicates.

Figure 6. Planktonic nitrosative stress assay comparing growth of UAMS-1, *nor* mutant, *hmp* mutant, and *nor hmp* mutants

The OD₆₀₀ of low-oxygen planktonic cultures grown at 37°C for a 24 hour period were read every hour with a Biotek Synergy HT fluorescent microplate reader. Cultures were either treated with 2 mM DPTA/NO at time of inoculation (A) or left untreated at the time of inoculation (B). All data represent the average $\pm$ SEM of n=4 biological replicates acquired over 2 independent experiments.

Figure 7. Contribution of Hmp and saNOR to nitrosative stress resistance during anaerobic growth

A: UAMS-1 and indicated isogenic mutants were grown under low-oxygen conditions (0 RPM, ambient atmosphere) in a 24-well plate in the absence or presence of DPTA/NO (0.25 mM, 0.5 mM, 1.0 mM, or 2.0 mM final concentration) added at the time of inoculation. The OD₆₀₀ of each well (culture) was measured after 12 hours growth using a microplate reader.

B: Experiment was conducted as described for A (above), except that plates were cultured anaerobically using an Anoxomat anaerobic system (Mart Microbiology, The Netherlands). For A and B, data represent the average of n=5 independent experiments ± SEM. *denotes statistical significance relative to UAMS-1 within each control (untreated) or treatment condition.

Figure 8. Contribution of saNOR to *S. aureus* respiration assessed by CTC staining

A: Cells were harvested from 9 hour planktonic low-oxygen cultures of UAMS-1 pMK4, KB1031 pMK4 (*nor* mutant), and KB1031 pMKnor (*nor* complement), washed with PBS and stained with 4.5 mM CTC. Cultures were either left untreated or treated with 2 mM DPTA/NO at time of culture inoculation. Fluorescence (RFU) was measured after 70 minutes of CTC staining with a Biotek Synergy HT fluorescent microplate reader was normalized to the initial OD₆₀₀ reading of each sample. *denotes statistical significance ($p < 0.001$ Holm-Sidak method) relative to UAMS-1 treated with 2 mM DPTA/NO. **denotes statistical significance ($p < 0.05$, Tukey Test) relative to untreated UAMS-1. **B:** Cells were harvested from 9 hour planktonic low-oxygen cultures of LAC-13C, LAC-13C (pMK4), and LAC-13C (pMKnor; “LAC-*nor*+”), washed and stained with 4.5 mM CTC as described above. Cultures were either left untreated or treated with 2 mM DPTA/NO at time of culture inoculation, and RFU/OD₆₀₀ measurements were obtained as described above. *denotes statistical significance relative to both the untreated LAC-13C wild type and LAC-13C (pMK4) ($p < 0.05$, Student-Newman-Keuls Method) **denotes statistical significance relative to both the NO-treated LAC-13C and LAC-13C (pMK4) ($p < 0.05$, Tukey test). For both graphs, data represent the average $\pm$ SEM of $n=3$ independent experiments.

Figure 9. Effect of NO and saNOR on membrane potential

Cultures were grown in planktonic low-oxygen conditions for 9 hours at 37°C. Cells were then harvested, washed and diluted in PBS, stained in 30 μ M of the membrane potential stain DiOC₂(3), and subjected to flow cytometry to detect the ratio of red to green fluorescence. Cultures were either left untreated or treated with 2 mM DPTA/NO at time of culture inoculation. Histograms represent the ratio of red to green fluorescence (X axis) in untreated (thick grey line histogram) and 2mM DPTA/NO treated (thin black line histogram) culture conditions, plotted against the number of events (Y axis). Data are representative of n=4 biological replicates acquired over 2 independent experiments. The average of each median value of red/green fluorescence ratios $\pm$ SEM are as follows: UAMS-1 untreated 104226.5 ± 11271.6 and 2mM DPTA/NO treated 142680.3 ± 2489.8 , *nor* mutant untreated 99746.3 ± 11245.4 and 2 mM DPTA/NO treated 107763.5 ± 3674.7 , and *nor* complement untreated 99466.8 ± 9673.2 and 2 mM DPTA/NO treated 141983.3 ± 8528.5 . The red/green fluorescent ratio of the NO-treated *nor* mutant is significantly different compared to the NO-treated UAMS-1 strain ($p < 0.05$, Dunnett's Method).

Table 1 Genome-sequenced strains of *S. aureus* that contain the *nor* gene.

<i>nor</i> + <i>S. aureus</i> Strain	Sequence Type	Methicillin Sensitivity	References	<i>nor</i> gene locus tag
TCH60	---	MRSA	Direct sequence submission to NCBI	HMPREF0772_10238
MRSA252	ST36	HA-MRSA/EMRSA-16	(Holden <i>et al.</i> 2004) (Nienaber <i>et al.</i> 2011)	SAR0261
Z172	ST239	HA-MRSA	(Chen <i>et al.</i> 2013)	SAZ172_0267
55/2053	---	MRSA	Direct sequence submission to NCBI	SAAG_00745
T0131	ST239	HA-MRSA	(Li <i>et al.</i> 2011)	SAT0131_00257
TW20	ST239	HA-MRSA	(Holden <i>et al.</i> 2010)	SATW20_02660
JKD6008	ST239	MRSA	(Howden <i>et al.</i> 2010)	SAA6008_00238
Bmb9393	ST239	HA-MRSA	(Amaral <i>et al.</i> 2005) (Costa <i>et al.</i> 2013)	SABB_05444
JKD6159	ST93	CA-MRSA	(Chua <i>et al.</i> 2010)	SAA6159_00241
LGA251	ST425	LA-MRSA	(Garcia-Alvarez <i>et al.</i> 2011)	SARLGA251_02280
ED133	ST133	LA-MSSA	(Guinane <i>et al.</i> 2010)	SAOV_0204
HO 5096 0412	ST22	HA-MRSA/EMRSA-15	(Holden <i>et al.</i> 2013)	SAEMRSA15_02230
71193	ST398	LA-MSSA	(Uhlemann <i>et al.</i> 2012)	ST398NM01_0277
08BA02176	ST398	LA-MRSA	(Golding <i>et al.</i> 2012)	C248_0252
ST398	ST398	LA/CA-MRSA	(Schijffelen <i>et al.</i> 2010)	SAPIG0277
H-EMRSA-15	---	EMRSA-15	Direct sequence submission to NCBI	ER16_01155
XN108	ST239	HA-MRSA/VISA	(Zhang <i>et al.</i> 2013)	SAXN108_0244
FORC_001	---	MSSA	Direct sequence submission to NCBI	FORC1_0200
ATCC 25923	---	MSSA	(Treangen <i>et al.</i> 2014)	KQ76_01055
GV69	---	MRSA	Direct sequence submission to NCBI	SAGV69_00298

HA: Hospital associated; CA: Community associated; LA: Livestock associated; ---: Unknown

Table 2

%Cells expressing *nor*-GFP fluorescence under different low-oxygen growth conditions.

Strain	Condition	%GFP cells Detected	
		Gate 1	Gate 2
Wild Type (UAMS-1) pMK_{nor}GFP	Biofilm	19.44 ± 1.70	1.31 ± 0.08
	Planktonic Biofilm Medium	28.59 ± 5.49	3.02 ± 0.62
	Planktonic TSB	15.88 ± 4.96	0.83 ± 0.09
<i>srrAB</i> mutant pMK_{nor}GFP	Biofilm	0.74 ± 0.03	0.06 ± 0.02
	Planktonic Biofilm Medium	1.46 ± 0.23	0.28 ± 0.12
	Planktonic TSB	0.86 ± 0.15	0.11 ± 0.09
Negative Control	Planktonic Biofilm Medium	1.26 ± 0.26	0.01 ± 0
Positive Control	Planktonic Biofilm Medium	97.05 ± 0.63	91.92 ± 1.87

The average (n=3 biological replicates acquired in two independent experiments) percentage of total GFP+ cells ± standard error of the mean (SEM) for each strain and GFP reporter plasmid is listed under “Gate 1”, and the average percentage of highly-fluorescent GFP+ cells ± SEM is listed under “Gate 2” (Gate numbers correspond to labels in Figure 6 and Figures S3-S4). All samples were harvested from 7 hour static biofilms, or from 7 hour planktonic low-oxygen cultures grown in either biofilm medium or TSB. Positive control = UAMS-1 pMKc/dGFP; Negative control = UAMS-1 pMK-GFP (promoterless).