The Response of *nor* and *nos* Contributes to *Staphylococcus aureus* Virulence and Metabolism

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ABSTRACT *Staphylococcus aureus* causes a wide spectrum of disease, with the site and severity of infection dependent on virulence traits encoded within genetically distinct clonal complexes (CCs) and bacterial responses to host innate immunity. The production of nitric oxide (NO) by activated phagocytes is a major host response to which *S. aureus* metabolically adapts through multiple strategies that are conserved in all CCs, including an *S. aureus* nitric oxide synthase (Nos). Previous genome analysis of CC30, a lineage associated with chronic endocardial and osteoarticular infections, revealed a putative NO reductase (Nor) not found in other CCs that potentially contributes to NO resistance and clinical outcome. Here, we demonstrate that Nor has true nitric oxide reductase activity, with *nor* expression enhanced by NO stress and anaerobic growth. Furthermore, we demonstrate that *nor* is regulated by MgrA and SrrAB, which modulate *S. aureus* virulence and hypoxic response. Transcriptome analysis of the *S. aureus* UAMS-1, UAMS-1 Δ *nor*, and UAMS-1 Δ *nos* strains under NO stress and anaerobic growth demonstrates that Nor contributes to nucleotide metabolism and Nos to glycolysis. We demonstrate that Nor and Nos contribute to enhanced survival in the presence of human polymorphonuclear cells and have organ-specific seeding in a tail vein infection model. Nor contributes to abscess formation in an osteological implant model. We also demonstrate that Nor has a role in *S. aureus* metabolism and virulence. The regulation overlap between Nor and Nos points to an intriguing link between regulation of intracellular NO, metabolic adaptation, and persistence in the CC30 lineage.

IMPORTANCE *Staphylococcus aureus* can cause disease at most body sites, and illness spans asymptomatic infection to death. The variety of clinical presentations is due to the diversity of strains, which are grouped into distinct clonal complexes (CCs) based on genetic differences. The ability of *S. aureus* CC30 to cause chronic infections relies on its ability to evade the oxidative/nitrosative defenses of the immune system and survive under different environmental conditions, including differences in oxygen and nitric oxide concentrations. The significance of this work is the exploration of unique genes involved in resisting NO stress and anoxia. A better understanding of the functions that control the response of *S. aureus* CC30 to NO and oxygen will guide the treatment of severe disease presentations.

KEYWORDS *Staphylococcus aureus*, metabolism, nitric oxide reductase, nitric oxide synthase, virulence

Staphylococcus aureus is an opportunistic pathogen with a clinical spectrum ranging from asymptomatic skin colonization to osteoarticular infections, endocarditis, and death (1, 2). It metabolically adapts to diverse environments within the human host, both as an extracellular pathogen and as an invasive pathogen of nonphagocytic cells

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(3, 4). The clinical presentation of disease is determined by a diverse repertoire of virulence and metabolic factors dispersed among eleven major clonal complexes (CCs), which are defined by genomic relatedness (5, 6). Although the majority of CCs have the capacity to cause disease, CC30 is strongly associated with mucosal colonization, hematogenous spread, and persistent, chronic infections (1, 5, 7–10). It produces less alpha-toxin than other clonal complexes due to a mutation in *hla* which renders it better able to colonize mucosal surfaces without quickly killing the host (11). While associated with the mucosa, CC30 can cause infections by producing toxic shock syndrome toxin 1 (TSST-1) and/or spreading from the mucosal surface; these processes feature regulation by the two-component systems that sense and respond to changes in oxygen levels (10, 11). CC30 strains are genetically more divergent than strains in other CCs, with genes unique to CC30 contributing to its distinct metabolic and clinical features (12, 13). Here, we assess the function of a recently identified gene, *nor* (nitric oxide reductase), encoding a putative nitric oxide reductase unique to CC30, its role in resistance to nitric oxide (NO), its relationship with *nos* (nitric oxide synthase), and increased persistence of this lineage within the mammalian host.

S. aureus resistance to phagocyte-derived nitric oxide (NO) is a highly unique trait and a major factor contributing to pathogenicity within the human host. At virtually all stages of infection, *S. aureus* controls the generation of host NO, which inhibits bacterial respiration and other functions through damage to metal enzyme centers, DNA, and proteins (14–18). *S. aureus* employs multiple strategies that are conserved among all CCs to counter the effects of NO, including detoxification of NO to N₂O (19–28) by a flavohemoprotein (Hmp) (19–21), induction of L-lactate dehydrogenase (Ldh1) to balance redox (29), induction of terminal oxidases to overcome inhibition of respiration (30, 31), and an active F₁F_o ATPase to maintain the alkaline cytosol required for NO resistance (32). In addition, all *S. aureus* strains produce endogenous NO via oxidation of L-arginine to L-citrulline by a nitric oxide synthase (Nos) (33–38). Endogenous NO provides protection against oxidative damage by interfering with Fenton chemistry (39), promotes aerobic respiration (40, 41), and mediates the switch to nitrate-based respiration during microaerobic growth (42).

The discovery of *nor* in CC30 raises the possibility of unidentified NO regulatory/modulation mechanisms that direct its physiological adaptation, likely in synergy with the conserved *S. aureus* NO response mechanisms. Detoxification of NO by Nor and Hmp may support increased persistence of CC30 in bacteremia and its significant association with osteomyelitis. The two anticipated opposing activities of Nor and Nos in *S. aureus* CC30 further suggest a potential relationship that modulates the intracellular concentration of NO in response to changes in host environmental O₂ and NO occurring during colonization and tissue invasion.

The genomic location of *nor* suggests potential roles in signaling, electron exchange, detoxification, or sensing of phagocytic products (43–48). Directly upstream of *nor* are *lrgA* and *lrgB* and their regulators, *lytR* and *lytS*, which together regulate extracellular murein hydrolysis, programmed cell death, aspects of biofilm formation, and sense perturbations in membrane potential caused by neutrophil by-products and innate immune effector molecules (44, 48). Following this logic, we demonstrate that *nor* is upregulated under anaerobic conditions and therefore serves as a potential link between oxygen sensing, NO, virulence, and metabolism in CC30 strains.

In this study, we found that *nor* and *nos* expression is induced by NO stress and anaerobic growth and that *nor* contributes to the elimination of NO in solution. Transcriptome analysis revealed that deletion of *nor* causes profound changes in nucleotide metabolism under NO stress and anaerobic growth, whereas deletion of *nos* impacts glycolysis under NO stress and nucleotide metabolism under anaerobic growth. Changes in gene expression identified by transcriptome data translated to an altered nucleotide metabolite profile under NO stress for strain UAMS-1 Δ *nor*. Assays using human polymorphonuclear cells (PMNs) suggest that Nor and Nos may both be involved in the survival of CC30 *S. aureus* in the presence of activated neutrophils. *In vivo* results show a role for Nor in osteological implant models of mouse

FIG 1 Function and expression of *nor*. (A) The rate of nitric oxide removal from solution is diminished in UAMS-1 Δnor . In cells complemented with *nor*, the time to reach half of the maximum NO concentration returns to wild-type levels. UAMS-1 Δhmp shows an exaggerated delay in the removal of nitric oxide from solution. The data are represented as minutes to reach half-maximum concentration $\pm$ standard deviations (SD) in ≥ 3 biological replicates. Significance was determined using one-way analysis of variance (ANOVA) with Dunnett's posttest (***, $P < 0.001$; ****, $P < 0.0001$). (B) *nor* is induced under anaerobic conditions. *nor* transcript levels normalized to *ftsZ* are shown as the fold induction in ≥ 3 biological replicates $\pm$ the standard errors of the mean (SEM). Significance was determined using one-way ANOVA with Tukey's posttest (**, $P < 0.01$; ***, $P < 0.001$). (C) Nitric oxide stress created by the addition of DEA/NONOate induces expression of *nor*. *nor* transcript levels normalized to *ftsZ* are shown as the fold induction in ≥ 3 biological replicates $\pm$ the SEM. Significance was determined using one-way ANOVA with Tukey's posttest (*, $P < 0.05$).

infection. Interestingly, we identify a possible organ-specific phenotype for Nor and Nos in CC30 *S. aureus* in a tail vein model of hematogenous infection.

RESULTS

***nor* contributes to the elimination of nitric oxide in solution.** CC30 *S. aureus* encode a putative quinol type nitric oxide reductase, designated locus SAR0261 in *S. aureus* MRSA252 (23, 24, 49). Its closest homolog is encoded by *Geobacillus stearothermophilus* (28, 50), predicted to share conserved amino acids, ligands, and cofactors and which has demonstrated NO reductase activity (27, 28). Despite this structural and predicted functional similarity demonstrated using fluorophore based cell permeable NO dyes (27), there is no direct, quantitative evidence for the function of *S. aureus* Nor on NO. To determine whether *nor* encodes a protein with NO reductase activity, we measured the ability of UAMS-1 and an isogenic *nor* deletion mutant to decrease concentrations of NO in a solution of NO donor diethylamine NONOate (DEA/NONOate; $t_{1/2} = 2$ min) (Fig. 1A). The time needed for a suspension of cells treated with 2 mM DEA/NONOate to eliminate half of maximum NO concentration is increased in UAMS-1 Δnor by $\sim 51\%$ compared to UAMS-1. The diminished ability of UAMS-1 Δnor to detoxify NO was restored by complementation with SAR0261 on a plasmid. Strain UAMS-1 Δhmp (*hmp*, flavohemoprotein gene), lacking a flavohemoprotein and previously shown to exhibit diminished NO removal, was included as a positive control (19–21). This is a critical demonstration of the nitric oxide reductase activity of the *nor* gene product measured by direct quantitative methods.

Anaerobic and nitric oxide stress induce expression of *nor*. We examined the expression of *nor* under conditions of anaerobiosis. Expression of *nor* begins to increase 1 h after anaerobic growth, peaks at >100-fold induction between 1 and 4 h, and then decreases (Fig. 1B). This is a much larger increase in *nor* expression than previously reported under conditions of hypoxia (27). Another novel finding is that NO stress increases *nor* expression, although to a lesser extent (Fig. 1C). Under anaerobic but not aerobic growth, UAMS-1 Δnor lags slightly in growth and final optical density (OD) compared to UAMS-1 (see Fig. S1A and B in the supplemental material). The effect of NO on long-term growth was measured by exposing bacteria to diethylenetriamine NONOate (DETA/NONOate; $t_{1/2}$ = 20 h). No significant growth inhibition was observed until 4 mM of DETA/NONOate was added (Fig. S1C). UAMS-1 and UAMS-1 Δnor did not differ in growth under these conditions (Fig. S1C). To confirm that the tested concentrations of DETA/NONOate produced a phenotypic change, UAMS-1 Δhmp was included as a control (Fig. S1D). Although anaerobicity and NO stress produce increased expression of *nor*, neither condition caused a growth defect in UAMS-1 Δnor . Possible interpretations are that under these conditions bacteria lacking *nor* have compensatory mechanisms to stabilize growth, such as Hmp, which is present in all CCs, or that *nor* contributes to growth under anaerobic conditions of nitrosative stress only (27).

MgrA and SrrAB regulate both *nor* and *nos* expression under NO stress and anaerobic growth conditions. To better understand the role of *nor* in anaerobic and NO stress conditions, we investigated the regulation of *nor* and *nos* expression. A preliminary promoter pulldown experiment using anaerobically grown and NO stressed UAMS-1 revealed potential regulatory proteins that bound the 500-bp region upstream of the *nor* transcription start site (data not shown). The two-component system SrrAB is particularly interesting due to its known role in regulating the *S. aureus* response to hypoxic stress (27, 51, 52). Therefore, we constructed an in-frame deletion of *srrB* and used quantitative reverse transcription-PCR (qRT-PCR) to further investigate its role in *nor* and *nos* regulation. In addition to SrrB, MgrA was identified as a potential regulator of *nor*; an in-frame *mgrA* deletion strain was created and evaluated side by side with the *srrB* deletion. Under NO stress, *nor* is not regulated by either SrrB or MgrA, but *nos* is activated by MgrA (Fig. 2A and B). After 2 h of anaerobic growth, both *nor* and *nos* expression is diminished in UAMS-1 $\Delta srrB$, confirming that SrrAB is a crucial activator of both genes under limited oxygen (27, 52) and establishing them as regulators following entirely anaerobic growth. Conversely, both *nor* and *nos* expression is enhanced in UAMS-1 $\Delta mgrA$, suggesting that MgrA inhibits these genes in the absence of oxygen.

Bacterial metabolism is affected by *nor* and *nos* at the transcript and metabolite levels under NO stress conditions. Transcriptome sequencing (RNASeq) was performed to analyze the relationship of NO stress, transcript abundance, and the presence of *nor* and *nos* in the UAMS-1 genome. Differences in the expression of metabolic pathways between strains were evaluated using the HUMAnN2 metabolic analysis network (53), which identifies pathway abundance as a function of the abundance of component reactions or genes in each pathway. We then modeled the pathway abundance data using a strategy that accounted for interaction between multiple experimental variables and identified interaction terms that describe the combined effect of NO and strain or of anaerobic growth and strain on pathway abundance (Fig. S2). This strategy allowed us to quantify the effect of multiple experimental variables on pathway abundance that could not be determined using pairwise comparisons of UAMS-1 and isogenic deletion strains alone. Briefly, reads from RNASeq were mapped to a staphylococcal pangenome, as well as the protein consensus sequence database using HUMAnN2, with >99% of all reads mapping to *S. aureus*. HUMAnN2 defined pathway abundance was used for principal component analysis and revealed that the data clustered into four groups, with biological replicates tending to group together within those clusters (Fig. 3). Bacteria not treated with DEA/NONOate or not undergoing anaerobic growth prior to RNASeq cluster together. Treatment with

FIG 2 *nor* and *nos* are regulated by *mgrA* and *srrB*. (A) Under nitric oxide stress conditions induced with DEA/NONOate, *nor* is not regulated by either *srrB* or *mgrA*. Data shown are mean relative fold change of *nor* transcript normalized to *ftsZ* in three independent replicates $\pm$ the SEM. Significance was determined using one-way ANOVA with Dunnett's posttest. (B) *nos* is activated by *mgrA* under NO stress conditions. Data show the mean relative fold changes in *nos* transcripts normalized to *ftsZ* in three independent replicates $\pm$ the SEM. Significance was determined using one-way ANOVA with Dunnett's posttest (**, $P < 0.01$). (C) *nor* is activated by *srrB* and repressed by *mgrA* after 2 h of anaerobic growth. Data show the mean relative fold changes in *nor* transcripts normalized to *ftsZ* in three independent replicates $\pm$ the SEM. Significance was determined using one-way ANOVA with Dunnett's posttest (***, $P < 0.001$; ****, $P < 0.0001$). (D) *nos* is activated by *srrB* and is repressed by *mgrA* after 2 hours of anaerobic growth. Data show the mean relative fold changes in *nos* transcripts normalized to *ftsZ* in three independent replicates $\pm$ the SEM. Significance was determined using one-way ANOVA with Dunnett's posttest (***, $P < 0.001$; ****, $P < 0.0001$).

2 mM DEA/NONOate, 120 min of anaerobic growth, and 240 min of anaerobic growth each form their own general groupings.

We next fit a linear regression model of the association between pathway abundance and experimental covariates (strain, treatment, and the interaction between strain and treatment) to identify significant interactions (54). In all cases, the linear models fit with a strong positive correlation (R^2 value of >0.8). NO treatment and strain had significant interactions associated with 11 pathways (Fig. 4A and Table S1). Analysis also revealed that when UAMS-1, UAMS-1 Δnor , and UAMS-1 Δnos were treated with 2 mM DEA/NONOate, 25 metabolic pathways were significantly up- or downregulated compared to untreated cells (Table S2). These 25 pathways were grouped into superclasses using MetaCyc (Fig. 4B) (33). Of particular interest is the increase in the purine *de novo* nucleotide biosynthesis superpathway. It is interesting that while the overall purine *de novo* biosynthesis superpathways, including both adenosine and guanosine, were significantly increased in UAMS-1, UAMS-1 Δnor , and UAMS-1 Δnos in the presence of nitric oxide stress, some individual subpathways, such as 5-aminoimidazole ribonucleotide and inosine-5'-phosphate biosynthesis, were significantly decreased under the same conditions and to different extents, depending on the strain. Furthermore, the expression levels of individual genes in the pathways differed between UAMS-1 and UAMS-1 Δnor . Selected genes from these pathways were validated using qRT-PCR (Table S3).

FIG 3 Principal component analysis of abundances of pathways present in UAMS-1, UAMS-1 Δnor , and UAMS-1 Δnos under the five conditions of RNASeq analysis: untreated with DEA/NONOate (NO $^-$)/untreated, aerobic growth (T0), treatment with DEA/NONOate (NO $^+$), 120 min of anaerobic growth (T120), and 240 min of anaerobic growth (T240).

To better understand how regulation of specific pathways influenced measurable phenotypes, such as nucleotide interaction, targeted metabolomics were performed on UAMS-1 and UAMS-1 Δnor . The results showed that a change in cellular concentrations of NMN, ADP, ATP, CDP, CTP, UMP, UTP, TDP, TTP, NADH, NADPH, and NAD/NADH occurs in UAMS-1 but not in UAMS-1 Δnor , or vice versa, in the presence of NO (Table 1). We observed that some nucleotide *de novo* synthesis pathways are upregulated in terms of transcript abundance but measured a decrease in metabolite synthesis, which could be explained by the allosteric regulation of nucleotide metabolism (55–57).

Bacterial metabolism is affected by *nor* and *nos* at the transcript and metabolite levels under aerobic and anaerobic growth. The same analysis described above was used to map RNASeq reads from UAMS-1, UAMS-1 Δnor , and UAMS-1 Δnos strains grown under anaerobic conditions. No statistically significant interaction terms were found when aerobically grown cells were compared to anaerobically grown cells. However, when we analyzed these pathways by strain and time alone, aerobically grown UAMS-1 Δnor upregulates pyruvate fermentation, UMP biosynthesis, inosine-5'-phosphate biosynthesis, and others but downregulates proline biosynthesis (Table S4). UAMS-1 Δnos upregulates fermentation and some glycolytic pathways while downregulating some purine *de novo* biosynthesis and pyrimidine salvage pathways. When we controlled for variation from aerobically grown cultures in our analysis, many interaction terms emerged to describe the relationship between the strain and the shift from 120 to 240 min anaerobic on pathway abundance (Table S5).

Global metabolomics were performed on UAMS-1 and UAMS-1 Δnor strains grown under aerobic and anaerobic conditions as defined in Materials and Methods. Unexpectedly, while 5,688 total metabolites were detected and searched against an internal retention time library of 1,100 known metabolites, only 60 were successfully identified (Table S6). Although this was too few metabolites for pathway analysis, many of the 60 individual metabolites were identified in the pairwise comparisons between UAMS-1 and UAMS-1 Δnor or aerobic and anaerobic conditions (Table 2).

Mutants deficient in *nor* and *nos* are more susceptible to neutrophil killing than UAMS-1. Since neutrophils are the primary innate immune cells that control acute

FIG 4 Pathways with significant change under NO stress conditions. (A) HUMAnN2 mapping identified 11 pathways whose abundance was uniquely impacted by the combination of strain and NO treatment. These pathways are said to have significant interaction. For pathways with significant interaction between strain and NO treatment that are unique to one strain, the increase (INC) or decrease (DEC) compared to the other two strains is noted. No pathways were unique to UAMS-1. No pathways were unique to UAMS-1 Δ *nor*. Three pathways were unique to UAMS-1 Δ *nos*: tetrapyrrole biosynthesis I from glutamate (DEC), glycolysis II from fructose-6-phosphate (DEC), and glycolysis (DEC). Three pathways show significant interaction in both UAMS-1 and UAMS-1 Δ *nor*: 5-aminoimidazole ribonucleotide biosynthesis II superpathway, 5-aminoimidazole ribonucleotide biosynthesis, and 5-aminoimidazole ribonucleotide biosynthesis II superpathway II. One pathway shows significant interactions between UAMS-1 and UAMS-1 Δ *nos*: UDP-*N*-acetyl-D-glucosamine biosynthesis I. Four pathways show significant interaction in all strains (UAMS-1, UAMS-1 Δ *nor*, and UAMS-1 Δ *nos*): superpathway of acetyl coenzyme A (acetyl-CoA) biosynthesis, selenoamino acid biosynthesis, adenosine ribonucleotides *de novo* biosynthesis, and coenzyme A biosynthesis I. All pathways are defined by MetaCyc via HUMAnN2, and significance is defined as a *P* value of <0.05 . A complete table of pathways with significant interactions under NO stress conditions can be found in Table S1. (B) HUMAnN2 mapping identified 31 pathways whose abundance was significantly ($P < 0.05$) changed by the presence of 2 mM DEA/NONOate compared pairwise with the untreated strain. These 31 pathways are grouped into MetaCyc superclasses for ease of viewing. Individual pathways are listed in Table S2. An analysis pipeline can be seen in Fig. S2.

S. aureus infection (58–60), we measured survival of *S. aureus* in the presence of PMNs. First, we determined that resting PMNs do not produce detectable levels of NO but that the addition of UAMS-1 activates the neutrophils and leads to the production of NO. Upon addition of the iNOS inhibitor *N*⁶-(1-iminoethyl)-L-lysine-dihydrochloride (L-NIL) to PMNs activated with UAMS-1, NO levels drop to resting state (Fig. 5A). We next established a target level of PMN killing and observed that UAMS-1 Δ *nor*, UAMS-1 Δ *nos*, and UAMS-1 Δ *hmp* are all more susceptible to PMN killing than is UAMS-1 (Fig. 5B). This increased susceptibility was previously shown by a *nos* deletion in a non-CC30 strain

TABLE 1 Targeted pyridine nucleotide and adenosine phosphate metabolomics

Nucleotide	UAMS-1 + NO vs UAMS-1 – NO		UAMS-1 Δ <i>nor</i> + NO vs UAMS-1 Δ <i>nor</i> – NO		UAMS-1 Δ <i>nor</i> – NO vs UAMS-1 – NO		UAMS-1 Δ <i>nor</i> + NO vs UAMS-1 + NO	
	% change	<i>P</i>	% change	<i>P</i>	% change	<i>P</i>	% change	<i>P</i>
NMN							–64.3	0.012
ADP	–67.8	0.034						
ATP	–82.0	0.024						
CDP	–47.1	0.042						
CTP	–72.4	0.040						
UMP							–51.5	0.046
UTP	–61.7	0.032						
TDP	–41.9	0.046						
TTP	–76.6	0.017						
NADH			–74.0	0.004				
NADPH	105.5	0.041						
NAD/NADH			167.7	0.011	–59.9	0.005		

TABLE 2 Global metabolomics of UAMS-1 and UAMS-1 Δnor under anaerobic growth conditions

Data set	No. of significant metabolites ($P < 0.05$)		
	Identified	Unknown	Total
UAMS-1 aerobic vs UAMS-1 anaerobic	6	449	455
UAMS-1 Δnor aerobic vs UAMS-1 Δnor anaerobic	42	588	600
UAMS-1 anaerobic vs UAMS-1 Δnor anaerobic	26	749	778

(39). Complementation with *nor*, *nos*, and *hmp* restores survival to wild-type levels (Fig. 5C). The addition of L-NIL causes an increase in the survival of all strains. Taken together, these results suggest that iNOS produced NO contributes to the killing of all tested bacterial strains and that the gene products of *nor*, *nos*, and *hmp* contribute to the survival of this killing. Our metabolomics analysis of UAMS-1 and UAMS-1 Δnor under nitrosative stress conditions indicated that UAMS-1 Δnor is deficient in NMN and UMP (Table 1). When NMN and UMP were added to the survival assay, UAMS-1 Δnor returned to wild-type survival (Fig. 5D and E). In a PMN-free suspension, UAMS-1 Δnor and UAMS-1 Δnos were not more susceptible to exogenous NO than UAMS-1, although UAMS-1 Δhmp was (Fig. 5F). In contrast, only UAMS-1 Δnos was more susceptible to exogenous H_2O_2 killing than any other strain (Fig. 5G).

***nor* and *nos* contribute to specific organ colonization in a tail vein injection model of hematogenous seeding.** To investigate whether *nor* or *nos* contributes to the ability of UAMS-1 to colonize distinct body sites via hematogenous seeding, we used a tail vein model of infection. UAMS-1, UAMS-1 Δnor , and UAMS-1 Δnos were injected singly in a bolus directly into the blood stream of CD-1 Swiss mice. In the kidney, the presence of *nor* in the genome or in complement provided an ~ 10 -fold increase in colonization compared to UAMS-1 Δnor (Fig. 6A). In the liver, the presence of *nos* in the genome or complement provided an ~ 10 -fold increase in colonization compared to UAMS-1 Δnos (Fig. 6B). In the lung, UAMS-1, UAMS-1 Δnor , and UAMS-1 Δnos all colonized to similar levels (Fig. 6C). These results suggest an organ-specific phenotype for both *nor* and *nos* in this model. When equal amounts of UAMS-1 and UAMS-1 Δnor were injected together into the same mouse, UAMS-1 Δnor had an ~ 10 -fold defect in colonization of all organs tested (Fig. 6D). Cytokine analysis of serum collected from mice infected with UAMS-1 or UAMS-1 Δnor at the time of sacrifice indicate that mice infected with UAMS-1 Δnor produce significantly less granulocyte colony-stimulating factor (G-CSF) than those infected with UAMS-1 (Fig. S3).

***nor* affects the ability to establish a soft tissue infection surrounding an osteological surgical implant.** In addition to hematogenous spread, CC30 strains of *S. aureus* are frequently the source of osteomyelitis (1, 5, 7–9). An osteological surgical implant was coated with either UAMS-1 or UAMS-1 Δnor and implanted in murine tibias. Although preliminary experiments did not show a significant difference in bone marrow abscesses between the two strains (data not shown), UAMS-1 caused a soft tissue infection surrounding the site of implantation with ~ 10 -fold-higher CFU compared to UAMS-1 Δnor (Fig. 7A). In addition, the proinflammatory cytokine IL-1 β was increased in sera from mice implanted with UAMS-1-coated surgical devices. Interleukin-4 (IL-4) and IL-2 were also increased in wild type-infected mice, whereas keratinocyte chemoattractant (KC) was enhanced in UAMS-1 Δnor infections (Fig. 7B to E).

DISCUSSION

In this study, we provide direct, probe-based evidence that *S. aureus* Nor functions to reduce the levels of NO in solution. The time required to eliminate half of peak NO concentration was significantly increased in a UAMS-1 Δnor strain. We also demonstrate that *nor* expression is induced by NO stress and anaerobic growth. We confirm previous work that SrrAB is an activator of *nor* (27) under anaerobic conditions, as well as an

FIG 5 Bacterial survival of nitrosative and oxidative stress in human PMNs and *in vitro*. (A) Freshly isolated human PMNs do not produce NOx levels above the limit of detection. When opsonized UAMS-1 is added to activate the PMNs, nitric oxide is produced. The addition of the iNOS inhibitor L-NIL blocks detectable levels of NOx. The data show the mean NOx concentrations $\pm$ the SD in ≥ 3 independent biological replicates. Significance was determined using one-way ANOVA with Dunnett's posttest (**, $P < 0.01$). (B) The percent survival of bacteria in human PMNs after 30 min is impacted by the NO metabolism genes *nor*, *nos*, and *hmp*. The data show the mean percent survival of bacteria $\pm$ the SD and represent ≥ 3 independent biological replicates. Significance was determined using one-way ANOVA with Dunnett's posttest (****, $P < 0.0001$). (C) Relative to UAMS-1, the UAMS-1 Δnor , UAMS-1 Δnos , and UAMS-1 Δhmp strains demonstrate decreased survival in the presence of human PMNs. This phenotype is ablated using gene complemented strains. When iNOS inhibitor L-NIL is added, all bacterial strains have a significantly increased percent survival compared to conditions without L-NIL. The data show mean percent survival relative to UAMS-1 $\pm$ the SD and represent ≥ 3 independent biological replicates. Significance was determined using two-way ANOVA with Tukey's posttest (**, $P < 0.01$; ****, $P < 0.0001$). (D) The addition of NMN to media during a killing assay demonstrates rescue of UAMS-1 Δnor to wild-type survival levels. Data show the mean relative percent survival $\pm$ the SD and represent ≥ 3 independent biological replicates. Significance was determined using one-way ANOVA with Dunnett's posttest (**, $P < 0.01$; ****, $P < 0.0001$). (E) The addition of UMP to media during a killing assay improves the survival of UAMS-1 Δnor to wild-type levels. Data show the mean relative percent survival $\pm$ the SD and represent ≥ 3 independent biological replicates. Significance was determined using one-way ANOVA with Dunnett's posttest (***, $P < 0.001$; ****, $P < 0.0001$). (F) In the absence of PMNs, UAMS-1 Δhmp has decreased survival when exposed to exogenous nitric oxide. Data show the mean relative percent survival $\pm$ the SD and represent ≥ 3 biological replicates. Significance was determined using one-way ANOVA with

(Continued on next page)

FIG 6 *nor* and *nos* contribute to hematogenous spread and organ specific seeding. (A) UAMS-1 Δnor , but not UAMS-1 Δnos , is significantly diminished in its ability to colonize the kidney following tail vein injection in mice. Data represent the mean CFU/gram of kidneys $\pm$ the SD in three separate experiments. Significance was determined using one-way ANOVA with Dunnett's posttest (*, $P < 0.05$). (B) UAMS-1 Δnos , but not UAMS-1 Δnor , is significantly diminished in its ability to colonize the liver following tail vein injection in mice. Data represent the mean CFU/gram of liver $\pm$ the SD in three separate experiments. Significance was determined using one-way ANOVA with Dunnett's posttest (***, $P < 0.001$). (C) Neither UAMS-1 Δnor or UAMS-1 Δnos is diminished in its ability to colonize the lungs following tail vein injection in mice. Data represent the mean CFU/gram of lungs $\pm$ the SD in three separate experiments. Significance was determined using one-way ANOVA with Dunnett's posttest. (D) UAMS-1 Δnor has a decreased competitive index in coinfections with UAMS-1 of ~ 10 -fold in the kidney, liver, and lungs.

activator of *nos* (52). In addition, we find that MgrA is a repressor of both *nor* and *nos* during anaerobic growth. Under nitrosative stress, neither *nor* nor *nos* is regulated by SrrB, but *nos* is activated by MgrA. SrrAB is an oxygen/nitrosative stress sensor and regulator required for virulence and growth of *S. aureus* during a shift from aerobic to anaerobic metabolism (51, 61). SrrAB is activated in environments of low molecular oxygen and in turn activates the transcription of NO detoxification systems, such as *hmp* (51) and, as we confirm in this study, for CC30 *nor* (27). The global regulator MgrA has been shown to regulate the expression of more than 350 genes and to be involved in virulence (62). More importantly, *mgrA* expression is autoregulated by its own oxidation sensing mechanisms (62), suggesting a link between MgrA and Nor. This could explain why we found that MgrA represses *nor* and *nos* under conditions of anaerobic growth but not during NO stress.

S. aureus is among the few Gram-positive bacteria that contain, in addition to *nor*, *nos*, which encodes a NO synthase (35, 38, 63–67). The formation of NO via Nos provides *S. aureus* with protection against oxidative damage by interfering with Fenton chemistry (39). To begin to evaluate the relationship between these genes and gene products and any effect such a relationship may have on *S. aureus*, we used RNASeq to map pathways with significant interaction between *nos*, *nor*, NO stress, and anoxia. The presence of *nos* in the genome led to significant interaction between *nos* and NO stress in three pathways, two of which pertain to glycolysis. Although we did not find any pathways with significant interaction unique to UAMS-1 Δnor in the presence of nitric

FIG 5 Legend (Continued)

Dunnett's posttest (***, $P < 0.001$). (G) In the absence of PMNs, UAMS-1 Δnos has decreased survival when exposed to exogenous H_2O_2 . Data show the mean relative percent survival $\pm$ the SD and represent ≥ 3 biological replicates. Significance was determined using one-way ANOVA with Dunnett's posttest (*, $P < 0.01$).

FIG 7 *nor* is involved in formation of an abscess surrounding an osteological surgical implant. (A) UAMS-1 Δnor formed abscess with tenfold fewer CFU compared to UAMS-1. Data show the mean CFU/abscess $\pm$ the SD (unpaired two-way *t* test; ****, $P < 0.0001$). (B) serum cytokine levels are significantly impacted by *nor*. Data show the mean $\pm$ the SD from ≥ 3 individual mice. Significance was determined using one-way ANOVA with Dunnett's posttest (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

oxide, it is important to note that this does not preclude important differences; for example, UAMS-1 and UAMS-1 Δnos both exhibit significant interaction between NO stress and the UDP-*N*-acetyl-D-glucosamine biosynthesis I pathway, whereas UAMS-1 Δnor did not. It is reasonable to infer that *nor*, which is present in both UAMS-1 and UAMS-1 Δnos , contributes to this change in the pathway. We find that NO stress upregulates pathways of purine and pyrimidine *de novo* synthesis in all three strains: UAMS-1, UAMS-1 Δnor , and UAMS-1 Δnos . However, when these pathways are examined in more specific detail, we find that, compared to UAMS-1, UAMS-1 Δnor significantly enhances the expression of the pathways that produce 5-aminoimidazole ribonucleotides, which are precursors to purine *de novo* biosynthesis.

Data from metabolomics experiments confirm that strain-specific changes in nucleotide transcript levels are also observed as differences in metabolite levels. In UAMS-1 cells but not UAMS-1 Δnor , levels of many nucleoside phosphates, including ATP, TTP,

UTP, and CTP, are decreased in the presence of NO. Decreased levels of NMN and UMP were found in NO-treated UAMS-1 Δnor cells compared to UAMS-1, and this suggests that *nor* may contribute to a metabolic redox change in the presence of NO. CTP, which is decreased in NO-treated UAMS-1 compared to UAMS-1 Δnor , is the allosteric inhibitor of pyrimidine metabolism (56, 57, 68). The decrease in CTP and ATP likely contributes to the increase in transcription of *pyrB* observed in RNASeq under the same conditions. The first committed step in purine *de novo* biosynthesis is catalyzed by *purF* and is inhibited in part by IMP. Since IMP was not found to be deficient in either UAMS-1 or UAMS-1 Δnor under NO stress conditions, this could explain the transcriptional repression of the IMP pathway identified by RNASeq results. Although it is unclear how *nor* effects nucleotide metabolism, there is no doubt that it profoundly impacts these pathways.

RNASeq was also performed on UAMS-1, UAMS-1 Δnor , and UAMS-1 Δnos strains grown aerobically, anaerobically for 120 min, and anaerobically for 240 min based on empirically determined time points of high *nor* expression. No significant interactions between strain and pathway were found when aerobically grown cells were compared to anaerobically grown cells. The results suggest that in the absence of oxygen, NO poses a minimal threat to *S. aureus*. However, when the transcripts from strains grown under aerobic conditions were compared, UAMS-1 Δnor showed increased levels of UMP biosynthesis pathway, lactate and acetate fermentation, and 5-aminoimidazole ribonucleotide biosynthesis compared to UAMS-1. UAMS-1 Δnos showed even lower abundances of UMP biosynthesis than UAMS-1, as well as purine and pyrimidine biosynthetic and salvage pathways, suggesting that endogenous levels of NO and the presence of oxygen are both important in regulating glucose metabolism and nucleotide energetics. In addition, when the shift from 120 to 240 min of anaerobic growth is considered while controlling for differences due to aerobic growth, interacting terms are found for many pathways, from glucose metabolism to cell wall biosynthesis, possibly showing that as the cells move into stationary phase in the absence of oxygen, *nor* and *nos* govern divergent elements of metabolism. Global metabolomics of UAMS-1 and UAMS-1 Δnor after aerobic growth and 120 min of anaerobic growth also yielded numerous strain- and condition-specific differences in the metabolite profile.

CC30 *S. aureus* has been linked to hematogenous spread, and we predicted that *nor* contributed to seeding of distant organs. Indeed, the competitive index of UAMS-1 Δnor has a 10-fold defect compared to the UAMS-1 in the kidney, lungs, and liver. This suggests that *nor* contributes a metabolic fitness advantage under certain conditions, possibly in the form of enhanced survival in blood or Trojan horse neutrophils (69, 70). Measured CFU of UAMS-1 were higher per gram of kidney than for any other tissue; this could indicate a preference of *S. aureus* for renal tissue once it has left the bottleneck of the liver (71). In single-strain infections, we observed a significant decrease in UAMS-1 Δnor CFU recovered from the kidney and UAMS-1 Δnos recovered from the liver; all strains colonized the lung equally. This indicates an organ-specific niche for *nor* and *nos* that is not due to a simple colonization defect. In the tail vein model, only G-CSF levels were significantly different in mice infected with UAMS-1 compared to UAMS-1 Δnor , possibly indicating that *nor* impacts induction of granulocyte production and neutrophil maturation.

S. aureus is a leading cause of infection on osteological implants, and CC30 has previously been implicated in osteomyelitis (1, 72, 73). We utilized a bone pin model of infection and found that surgical implants coated with bacteria resulted in soft tissue abscesses surrounding the bone; UAMS-1 caused abscesses with >10-fold more CFU than did UAMS-1 Δnor . Mice with UAMS-1-coated implants produced more IL-1 β , IL-2, and IL-4, while KC was elevated in UAMS-1 Δnor . IL-1 β is known to increase in response to *S. aureus* infection, as well as in osteomyelitis specifically (74, 75), and high IL-2 levels are also associated with *S. aureus* infections (76). Elevated levels of *S. aureus* infection associated cytokines in mice with a more robust *S. aureus* abscess are consistent with a host response proportional to the severity of the bacterial infection (77, 78).

In this study, we established a measurable function of *nor* on NO and defined

conditions resulting in increased expression, altered metabolic pathways, and changed metabolite profiles. It is tempting to speculate based on RNASeq and metabolomics that in wild-type cells treated with nitric oxide, the pentose phosphate pathway favors its oxidative stage rather than the nonoxidative stage, resulting in a dramatic increase in NADPH and decrease in phosphonucleotides. At the transcript level, genes involved in nucleotide biosynthesis are increased in response to the feedback loop of decreased levels of nucleotides. When *nor* is deleted, bacteria no longer exhibit this profile; we see an increased ratio of NAD⁺/NADH, suggesting an increased requirement for glycolysis in UAMS-1 Δnor (79, 80). Finally, we demonstrate with *in vivo* models the importance of *nor* and *nos* in pathogenesis. Although more work is needed to determine what role these nucleotide differences have on the observed differences seen in pathogenesis, there remains little doubt that *nor* is a contributing player to the pathogenesis of CC30 *S. aureus*, and its relationship with *nos* will prove to better instruct our understanding of the interplay of bacterial factors during infection.

MATERIALS AND METHODS

Strains. All of the strains used in this study are described in Table S7 in the supplemental material.

Genetic manipulation. The *Escherichia coli-Staphylococcus aureus* shuttle vectors pWedge and pKOR1 were used in the construction of in-frame deletions of *nor*, *nos*, and *hmp* in *S. aureus* UAMS-1 by allelic exchange, as previously described (81, 82). The deletions were confirmed by targeted or genome sequencing. Gene complement constructs were created by ligating the full-length deleted gene into pCN-40-erm (83).

NO detection and chemicals. Diethylamine NONOate (DEA/NONOate; $t_{1/2}$ = 2 min) and diethylene-triamine NONOate (DETA/NONOate; $t_{1/2}$ = 20 h) were used for short- and long-term NO exposure experiments, respectively. Both compounds were suspended in 10 mM NaOH at 500 mM, stored at -80°C , and diluted to 2 mM immediately prior to use. Experimental detection of NO was accomplished with an ISO-NOP Mark II electrode and isolated nitric oxide meter (WPI Instruments, Sarasota, FL). All experiments were performed in biological triplicate.

Acute NO stress conditions. The optimal concentration of DEA/NONOate donor for acute stress was determined by selecting the lowest concentration that produced increased expression of *hmp* without killing UAMS-1 and set at 2 mM. For experiments analyzing the effects of acute NO stress, UAMS-1 and isogenic mutants were grown overnight in broth and diluted 1:100 into target media. Cultures were grown at 37°C with shaking to early exponential phase (OD at 600 nm [OD₆₀₀] = 0.5) in a volume/flask ratio of 1:5, treated with 2 mM DEA/NONOate, and incubated at 37°C with shaking for 20 min.

Anaerobic stress conditions. Optimal anaerobic conditions were determined by monitoring *nor* expression over time. UAMS-1 and isogenic mutants were grown at 37°C with shaking to early exponential phase (OD₆₀₀ = 0.5). Cultures were then transferred to 50-ml Falcon tubes with no head space tightly sealed and then incubated at 37°C with shaking for 120 or 240 min in sealed GasPaks (BD, Franklin Lakes, NJ). Anaerobiosis was verified using 0.001% resazurin.

Human PMN survival. Human neutrophils were isolated from blood of healthy donors as previously described (39, 84) seeded at 1×10^6 cells/ml, allowed to adhere for 30 min, and infected with opsonized bacteria grown statically overnight in RPMI 1640 (Gibco) at a multiplicity of infection of 2. Identical control wells without PMNs were included side by side to calculate the percent survival. Bacteria were then centrifuged onto neutrophils and incubated at 37°C in 5% CO₂ for 30 min. After 30 min, neutrophils were lysed with saponin. NMN and UMP were added at 1 mM to wells containing bacteria and PMNs where indicated. Then, 50 μM L-NIL was added to the PMNs during the adherence step where indicated. A Griess reaction (Promega) was performed in triplicate to evaluate nitric oxide production and the efficacy of L-NIL under these conditions.

Targeted and global metabolomics. Targeted and global metabolomics processing was performed by the Southeast Center for Integrated Metabolomics at the University of Florida. Bacterial cell pellets from acute NO stress cultures were lyophilized overnight and processed as previously described (40). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to quantitate metabolites from cell homogenates and extracellular media. Organic acids in homogenates and media were spiked with isotopically labeled internal standards, extracted in ethylacetate, and dried extracts were derivatized with *O*-benzylhydroxylamine using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide coupling chemistry according to prior studies. For the extraction of nucleotides, cell homogenate was spiked with isotopically labeled internal standard and centrifuged, and supernatants were filtered through an AcroPrep Advance 3K Omega filter plate (Pall Corporation) prior to LC-MS/MS analysis. Derivatized organic acids and nucleotides were quantitated as previously described (40). For all classes of metabolites, data for cell samples were normalized to the protein, whereas supernatant concentrations are reported in micromolar units. For global metabolomics, a Thermo Q-Exactive Orbitrap mass spectrometer with Dionex UHPLC and an autosampler were used to analyze samples prepared under aerobic and anaerobic conditions. All samples were analyzed as separate injections using positive and negative heated electrospray ionization with a mass resolution of 35,000 at m/z 200. Mobile phase A (formic acid in water) and mobile phase B (acetonitrile) were used on an ACE 18-ppp column (100 by 2.1 mm, 2 μm) at a flow rate of 350 $\mu\text{l}/\text{min}$ at 25°C to achieve separation.

H₂O₂ killing assays. UAMS-1 and isogenic mutants were grown overnight in tryptic soy broth (TSB) and diluted 1:100, allowed to grow shaking at 37°C to an OD₆₀₀ of 0.5, and normalized by the OD. Strains were treated with 0.75% fresh H₂O₂ for 30 min at 37°C and then treated with catalase (Sigma) to quench the reaction, which was incubated in the suspensions for an additional 30 min. Assays were performed in biological triplicates. Treatment concentration of hydrogen peroxide was determined by selecting the concentration that yielded approximately 50% survival of UAMS-1.

NO killing assays. UAMS-1 and isogenic mutants were grown overnight in TSB, diluted 1:100 in TSB, and allowed to grow with shaking at 37°C to an OD₆₀₀ of 0.5. All strains were then normalized by the OD. Cells were treated with 2 mM DEA/NONOate (Cayman Chemicals) for 20 min with shaking at 37°C. Experiments were performed in biological triplicates.

Tail vein hematogenous seeding model. All experimental procedures and handling of mice were approved by the University Committee on Animal Resources at the University of Rochester Medical Center. UAMS-1, UAMS-1 Δ nor, or UAMS-1 Δ nos were injected into Swiss CD-1 outbred female mice (Envigo) at 10⁷ CFU/animal in a 200- μ l bolus. Mice were sacrificed after 72 h by CO₂ asphyxiation and cervical dislocation. The kidneys, heart, liver, and lungs were removed and homogenized using Lysing Matrix A tubes and MP FastPrep-Prep-24 (MP Biomedicals). A competitive infection experiment was performed under the same conditions with UAMS-1 and UAMS-1 Δ nor, except that 5 $\times$ 10⁶ CFU of each strain were mixed for a final concentration of 1 $\times$ 10⁷ CFU and injected together into the same animal. The competitive index was calculated using the following formula: competitive index = (mutant output/wild-type output)/(mutant input/wild-type input).

Bone pin infection model. All experimental procedures and handling of mice were approved by the University Committee on Animal Resources at the University of Rochester Medical Center. Male C57BL/6J mice (Jackson Laboratories) were infected with an orthopedic implant coated with $\sim$ 2 $\times$ 10⁵ CFU of *S. aureus* UAMS-1 or UAMS-1 Δ nor as previously described (85). After sacrifice, soft tissue surrounding the implant site was removed and homogenized as described above. A minimum of three independent biological replicates were performed for all murine infection experiments.

Multiplex cytokine panels. Blood was collected by cardiac puncture in both the CD-1 mice infected with UAMS-1 or UAMS-1 Δ nor used in the tail vein injection model and the B57BL/6J mice used in the bone pin infection model. Serum was removed from freshly clotted blood and stored at -80°C . The Luminex 32-plex (Eve Technologies) was used to assay serum cytokine levels.

RNASeq. UAMS-1, UAMS-1 Δ nor, and UAMS-1 Δ nos were evaluated using whole transcriptome analysis. Each strain was subcultured 1:100 from overnight cultures into TSB at a 1:5 volume/flask ratio, grown to early exponential phase (OD₆₀₀ = 0.5), and then shifted to either anaerobic conditions or subjected to NO stress. Experiments were performed in biological duplicate. Cultures were placed in RNAProtect (Qiagen, Valencia, CA) at the appropriate endpoint. RNA was isolated by homogenization by mechanical lysis in Trizol by using Lysing Matrix B tubes and MP FastPrep-Prep-24 (MP Biomedicals) and purified using DirectZol columns (Zymo Research, Irvine, CA). Residual DNA was removed by using Ambion DNase Turbo (Thermo Fisher Scientific, Waltham, MA). cDNA libraries were constructed by using a TruSeq stranded mRNA library kit (Illumina, San Diego, CA), analyzed for quantity and quality, and sequenced on an Illumina HiSeq 2500 (Illumina). Sequencing reads were cleaned by using the Trimmomatic-0.32 workflow prior to being mapped to MRSA252 with STAR-2.4.2a. Cufflinks2.0.2 was then used with MRSA252 gene annotations to analyze differential gene expression with a 95% confidence interval.

Cleaned reads were processed with the HUMAnN2 pipeline (53) using an *S. aureus* pangenome nucleotide sequence and UniRef90 nonredundant protein sequence references, yielding computed functional *S. aureus* profiles. (53). HUMAnN2 was run with default settings using the native ChocoPhlAn pan-genome nucleotide database and the UniRef90 protein database, except the taxonomic profiling step was bypassed, and a preconstructed taxonomic profile was provided to the program which contained equal parts of all *Staphylococcus* species present in the ChocoPhlAn database. Based on the mapped reads and the protein hits, the HUMAnN algorithm is used to estimate the coverage and relative abundance of functional pathways and gene families. Differential relative abundance of functional pathways was assessed using the MaAsLin algorithm (54), with model selection disabled and otherwise default settings. All regression models were fit with untreated Δ nor as the base case (intercept) unless otherwise noted. For models fit using multiple strains, interaction terms between treatment and strain were included to identify strain-specific differences in the effects of treatment. Interaction terms were used to identify the effects of predictor variables, such as treatment, that depend on the value of other predictor variables, such as strain. Interaction terms would be significant when the effect of treatment is different for one strain than it is for another. In such cases, interpreting the effect of treatment requires the consideration of the main effect, the effect of treatment alone, plus the interaction term between the treatment and a given strain. Principal component analysis and visualization were performed using R version 3.3.3 on the arcsin square-root transformed relative abundances of all pathways present.

Data availability. All experimental metadata, RNASeq sequence reads, and differential gene expression values are publicly available through NCBI BioProject [PRJNA464411](https://doi.org/10.1128/JB.00107-19). All bacterial strains are available from the corresponding author.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00107-19>.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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L.J.F. and S.R.G. designed the study. L.J.F. and A.L.G. constructed bacterial strains, carried out all experimental studies and generated data. C.W.F. and R.A.M. provided expert guidance on murine tibia infection experiments. L.J.F. analyzed all data, including RNA-Seq and metabolomic data. L.J.F. and S.R.G. drafted the manuscript. All authors reviewed the final manuscript.

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