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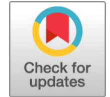
RESEARCH ARTICLE



Staphylococcus aureus Strain Newman D2C Contains Mutations in Major Regulatory Pathways That Cripple Its Pathogenesis

William E. Sause,[a] Richard Copin,[a] Aidan O'Malley,[a] Rita Chan,[a] Brian J. Morrow,[b] Peter T. Buckley,[c] Jeffrey Fernandez,[c] A. Simon Lynch,[c] Bo Shopsin,[a,d] Victor J. Torres[a]

Department of Microbiology, New York University School of Medicine, New York, New York, USA[a]; Janssen Research and Development, Raritan, New Jersey, USA[b]; Janssen Research and Development, Spring House, Pennsylvania, USA[c]; Department of Medicine, Division of Infectious Diseases, NYU School of Medicine, New York, New York, USA[d]

ABSTRACT *Staphylococcus aureus* is a major human pathogen that imposes a great burden on the health care system. In the development of antistaphylococcal modalities intended to reduce the burden of staphylococcal disease, it is imperative to select appropriate models of *S. aureus* strains when assessing the efficacy of novel agents. Here, using whole-genome sequencing, we reveal that the commonly used strain Newman D2C from the American Type Culture Collection (ATCC) contains mutations that render the strain essentially avirulent. Importantly, Newman D2C is often inaccurately referred to as simply "Newman" in many publications, leading investigators to believe it is the well-described pathogenic strain Newman. This study reveals that Newman D2C carries a stop mutation in the open reading frame of the virulence gene regulator, *agrA*. In addition, Newman D2C carries a single-nucleotide polymorphism (SNP) in the global virulence regulator gene *saeR* that results in loss of protein function. This loss of function is highlighted by complementation studies, where the *saeR* allele from Newman D2C is incapable of restoring functionality to an *saeR*-null mutant. Additional functional assessment was achieved through the use of biochemical assays for protein secretion, *ex vivo* intoxications of human immune cells, and *in vivo* infections. Altogether, our study highlights the importance of judiciously screening for genetic changes in model *S. aureus* strains when assessing pathogenesis or the efficacy of novel agents. Moreover, we have identified a novel SNP in the virulence regulator gene *saeR* that directly affects the ability of the protein product to activate *S. aureus* virulence pathways.

IMPORTANCE *Staphylococcus aureus* is a human pathogen that imposes an enormous burden on health care systems worldwide. This bacterium is capable of evoking a multitude of disease states that can range from self-limiting skin infections to life-threatening bacteremia. To combat these infections, numerous investigations are under way to develop therapeutics capable of thwarting the deadly effects of the bacterium. To generate successful treatments, it is of paramount importance that investigators use suitable models for examining the efficacy of the drugs under study. Here, we demonstrate that a strain of *S. aureus* commonly used for drug efficacy studies is severely mutated and displays markedly reduced pathogenicity. As such, the organism is an inappropriate model for disease studies.

KEYWORDS Newman, *Staphylococcus*, WGS, genome analysis, infection, pathogenesis, polymorphism, toxins, virulence

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Address correspondence to Victor J. Torres, victor.torres@nyumc.org.

R. Copin and A. O'Malley contributed equally to this work.

Staphylococcus aureus is a versatile human pathogen that is capable of causing a wide spectrum of disease states that can range from mild skin and soft tissue infections to life-threatening bacteremia (1). The pathogen has steadily taken a devastating toll on the health care system for decades, and in response, many inspired efforts have been undertaken to develop therapeutics capable of thwarting the bacterium's deadly effects (2). Concomitant with the demand to develop an effective antistaphylococcal therapy is the need to select the most applicable model(s) for studying disease caused by *S. aureus*. *S. aureus* strain Newman (here referred to as Newman) is a commonly used model strain for studying *S. aureus* pathogenesis (3–13) and in turn has been extensively used to examine the therapeutic efficacy of agents designed to treat *S. aureus* infection (12, 14–20). Newman was originally isolated from a human patient in 1952 (21, 22) and has since been a fixture in the investigation of *S. aureus* pathogenesis due in large part to its strong virulence in both animal models and human *ex vivo* systems.

In addition, Newman has served as a valuable measuring stick for assessing the therapeutic efficacy of agents designed to protect the host from *S. aureus* infection. The importance of Newman has been demonstrated by multiple studies in animals that have shown reduced S. *aureus*-caused lethality in cohorts treated with a number of different therapies (12, 14–20). In our own attempts to develop a therapeutic agent to treat *S. aureus* infection, we discovered significant inconsistencies in the virulence profiles of Newman strains and a derivative thereof, Newman D2C. Specifically, the strain Newman in our laboratory collection, which originates from the same source that was used in determining the original genome sequence (22), differed immensely in virulence compared to the strain Newman D2C obtained from the American Type Culture Collection (ATCC). Newman D2C was originally described as a clumping-factor-positive variant of Newman strain D2 (23), although the relationship between the original Newman strain and the Newman D2 strain is not apparent. As described here, Newman D2C from the ATCC was observed to exhibit reduced virulence in mouse models of infection and in *ex vivo* assays with human immune cells. This is in stark contrast to the widely used Newman strain described by Baba et al. (22), which is highly virulent in mice and highly cytotoxic to human tissues (3–13, 15, 24). Unfortunately, numerous studies utilize Newman D2C as a model strain for *S. aureus* virulence (25–31) and, in many cases, refer to the strain as simply "Newman" (25, 27, 31, 32). The use of this strain creates confusion and leads investigators to believe that it is the same strain originally isolated by Duthie et al. in 1952 (33) and sequenced by Baba et al. (22). This error is perhaps defensible, however, as the ATCC lists the chain of custody for Newman D2C as E. Duthie (1952) to J. Hawiger (1970) to the ATCC.

In an effort to understand the differences in virulence we observed between the strain of Newman in our laboratory collection and Newman D2C obtained from the ATCC, we performed whole-genome sequencing of the two strains. In doing so, we discovered multiple mutations in the ATCC strain compared to the strain in our collection. One mutation, a single-nucleotide variant, was identified in the DNA binding domain of the response regulator gene *saeR* of the *sae* regulatory system (34, 35). The *sae* locus contains four open reading frames (ORFs) (*saePQRS*), among which *saeS* encodes a two-component sensor histidine kinase and *saeR* encodes the cognate response regulator (34). The additional genes in the locus, *saeP* and *saeQ*, encode a less-well-characterized lipoprotein and a stabilization protein for SaeS, respectively (36, 37). The *sae* locus consists of two promoters (P1 and P3) that drive the production of four mRNA transcripts: the T1 transcript initiates upstream of *saeP* and extends the length of the *sae* locus, the T2 transcript starts in the intergenic region upstream of *saeQ*, transcript T3 begins directly upstream of *saeR*, and T4 only extends the length of *saeP* (38, 39). The *sae* regulatory system is critical for the expression and production of a number of key *S. aureus* virulence proteins and is essential for pathogenesis in animal models of infection (40–46). Here, we show that the *saeR* mutation in strain Newman D2C renders the *sae* system nonfunctional and thus incapable of activating virulence gene expression. We demonstrate that the *saeQRS* allele from the Newman

strain in our collection can rescue an *saeQRS*-null mutant, while the Newman D2C *saeQRS* allele is incapable of restoring pathogenesis in the same genetic background. Interestingly, a mutation in the *saeS* gene of strain Newman results in an L18P substitution in the first transmembrane domain and renders the sensor kinase constitutively active (47–49). Hence, the *saeR* mutation in strain Newman D2C nullifies the effects of the $SaeS^{L18P}$ protein (as found here in both strain Newman and Newman D2C). Moreover, this study raises concerns about the value of agents whose therapeutic efficacy has been assessed only with Newman D2C from the ATCC as a model organism and provides a cautionary note with regard to the future use of the strain in investigations that intend to examine *S. aureus* pathogenesis.

RESULTS

Strain Newman D2C is severely attenuated. In our collaborative efforts to develop novel antistaphylococcal protein therapeutics with Janssen R&D LLC (2), it was observed that the Janssen group was unable to reproduce virulence phenotypes routinely observed with Newman infections in mice (9–11). Upon investigating the source of their strain, it was determined that, unlike the origin of our strain (22), the group had obtained a strain referred to as Newman D2C from the ATCC. The ATCC lists this strain as having originated from the same source as Newman in our laboratory collection (21), and thus, it was deemed an appropriate model for conducting studies on *S. aureus* pathogenesis. The Newman in our collection, which we refer to below as "NYU Newman," originated from the strain that the genome sequence was obtained from (22). First, to corroborate the findings observed at Janssen, we examined the *in vivo* fitness level of Newman D2C compared to that of NYU Newman. Our results confirmed that the Newman D2C strain that Janssen acquired from the ATCC exhibited a significantly reduced ability to infect mice in multiple critical organs compared to NYU Newman (Fig. 1A). To confirm these observations, we obtained a fresh stock of Newman D2C from the ATCC and tested the strain's ability to induce lethality in mice. Consistent with the original study, we found that whereas the NYU Newman strain was acutely lethal to mice, the newly acquired Newman D2C was again found to be essentially avirulent (Fig. 1B).

In our efforts to assess the extent of Newman D2C's attenuation, we next screened for differences in hemolysis in Newman D2C compared to NYU Newman, as hemolysis is associated with *S. aureus* virulence (50). Using the method described by Adhikari et al. (51), which analyzes the synergistic hemolytic activity of β-hemolysin from RN4220 and δ-hemolysin from the *S. aureus* strain under examination, we found that Newman D2C was nonhemolytic while NYU Newman demonstrated hemolytic synergism between β-hemolysins, as well as α-hemolysin activity (Fig. 1C). Importantly, the δ-hemolysin synergism is a direct readout of Agr activity, an *S. aureus* regulatory system that is fundamental to the production of virulence products (46, 52–54). Thus, Newman D2C's lack of δ-hemolysin synergism serves as an indicator of Agr dysfunction in the strain.

Strain Newman is a member of the *S. aureus* clonal complex lineage 8 (CC8), a lineage known for secreting a multitude of cytotoxic virulence factors (46). To investigate whether there was an alteration in the levels of secreted proteins in Newman D2C, we took concentrated supernatants from *in vitro*-grown bacteria and examined global secretion profiles. Upon staining for all detectable secreted proteins, it was evident that Newman D2C had markedly reduced amounts of protein secretions compared to NYU Newman (Fig. 1D). To investigate whether the reduction in overall protein secretion corresponded to dampened production of key *S. aureus* secreted toxins, we also performed immunoblots using antibodies against either the LukB subunit of the LukAB leukotoxin (8, 55) or alpha-toxin (Hla) (56). In accordance with the exoprotein profiles, NYU Newman demonstrated clear toxin production while Newman D2C showed no apparent production of either toxin (Fig. 1D). Of note, the polyclonal anti-LukB antibody recognizes other homologous *S. aureus* leukotoxins (55), as indicated by the blot. As such, the lack of cross-reactivity in the Newman D2C supernatant supports the notion

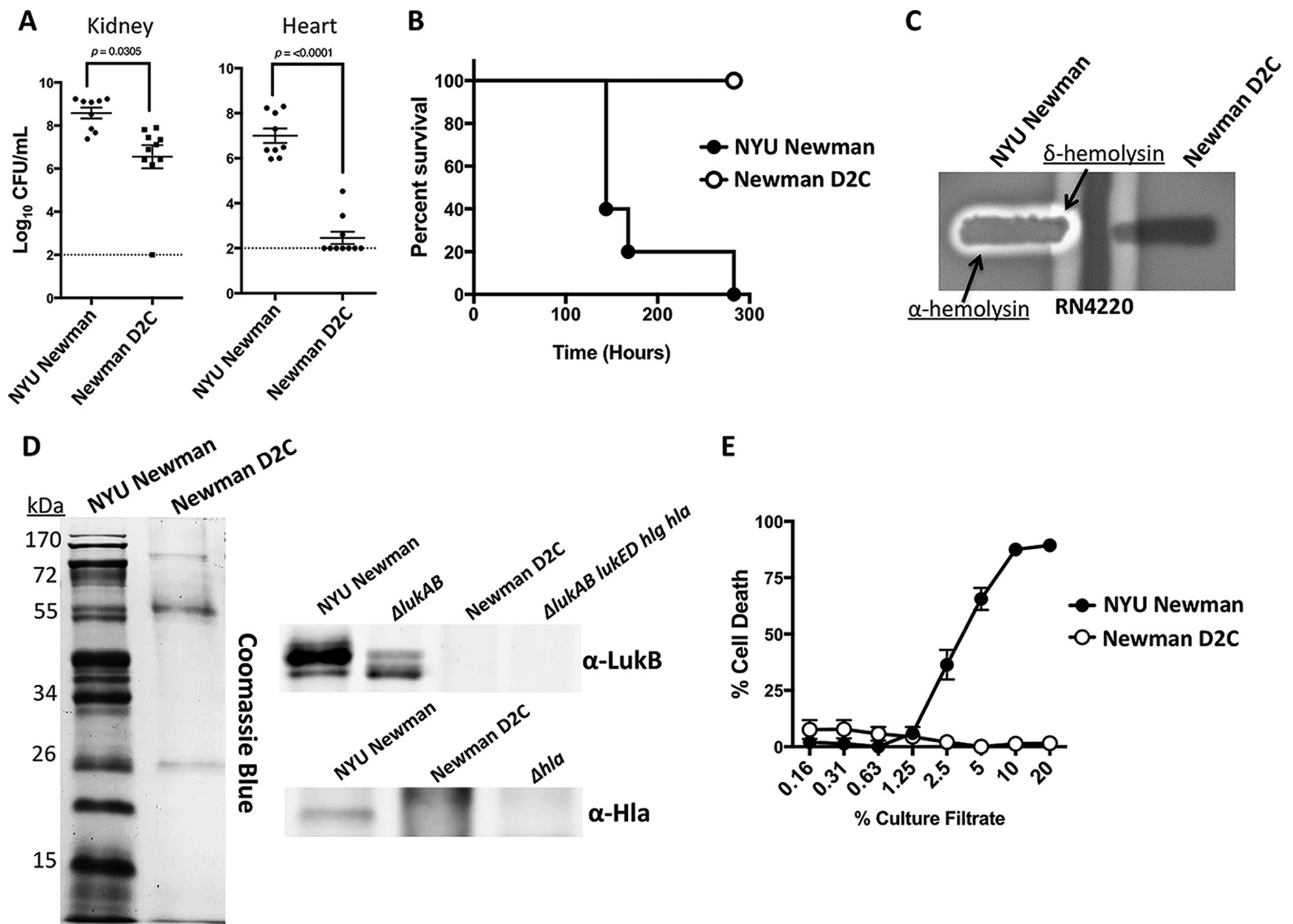

FIG 1 Newman D2C is nonpathogenic. (A) In an intravenous-infection model, *S. aureus* strain Newman D2C exhibited an attenuated ability to infect mice compared to that of NYU Newman ($n = 9$ and 10 mice/strain, respectively). The dashed lines indicate the limit of detection for the bacterial burden. Statistics were performed with Student's *t* test. (B) Newman D2C was nonpathogenic to mice in an assessment of lethality, while NYU Newman was acutely lethal ($n = 5$ mice). The points indicate individual mouse deaths. (C) Newman D2C was nonhemolytic in blood plates. (D) NYU Newman produced robust amounts of secreted proteins, whereas Newman D2C had minimal detectable proteins and no detectable levels of the toxin Hla or LukAB. (E) Culture filtrates from Newman D2C exhibited no observable cytotoxic properties against primary human PMNs, while NYU Newman was cytotoxic ($n = 3$ donors). The error bars indicate standard errors of the means.

that a global loss in toxin production had occurred. To further support this observation, we next examined the cytotoxicity of the two Newman strains on primary human polymorphonuclear leukocytes (PMNs). Consistent with the weak exoprotein profile displayed by Newman D2C, it was observed that culture filtrates obtained from Newman D2C were severely attenuated in cytotoxicity on primary PMNs (Fig. 1E). Taken together, these data support the notion that the attenuation observed in Newman D2C from the ATCC can be attributed to either a lack of key virulence factors or a defect in the regulatory machinery necessary to secrete these toxins.

Whole-genome sequencing identifies mutations within Newman D2C. To obtain a global picture of the genomic differences between NYU Newman and Newman D2C that could account for the gross phenotypic inconsistencies observed between the two strains, we performed whole-genome sequencing and compared both genomes to the Newman reference genomes available at the National Center for Biotechnology Information (NCBI), LT598688 and AP009351 (the NYU Newman parent strain [22]). After excluding mobile genetic elements, we identified a total of 143 single-nucleotide polymorphisms (SNPs) across the 4 genomes (Fig. 2). Among these, the Newman D2C genome contained 9 unique mutations (see Table S1 in the supplemental material). Interestingly, a handful of these mutations occur in genes that are instrumental in

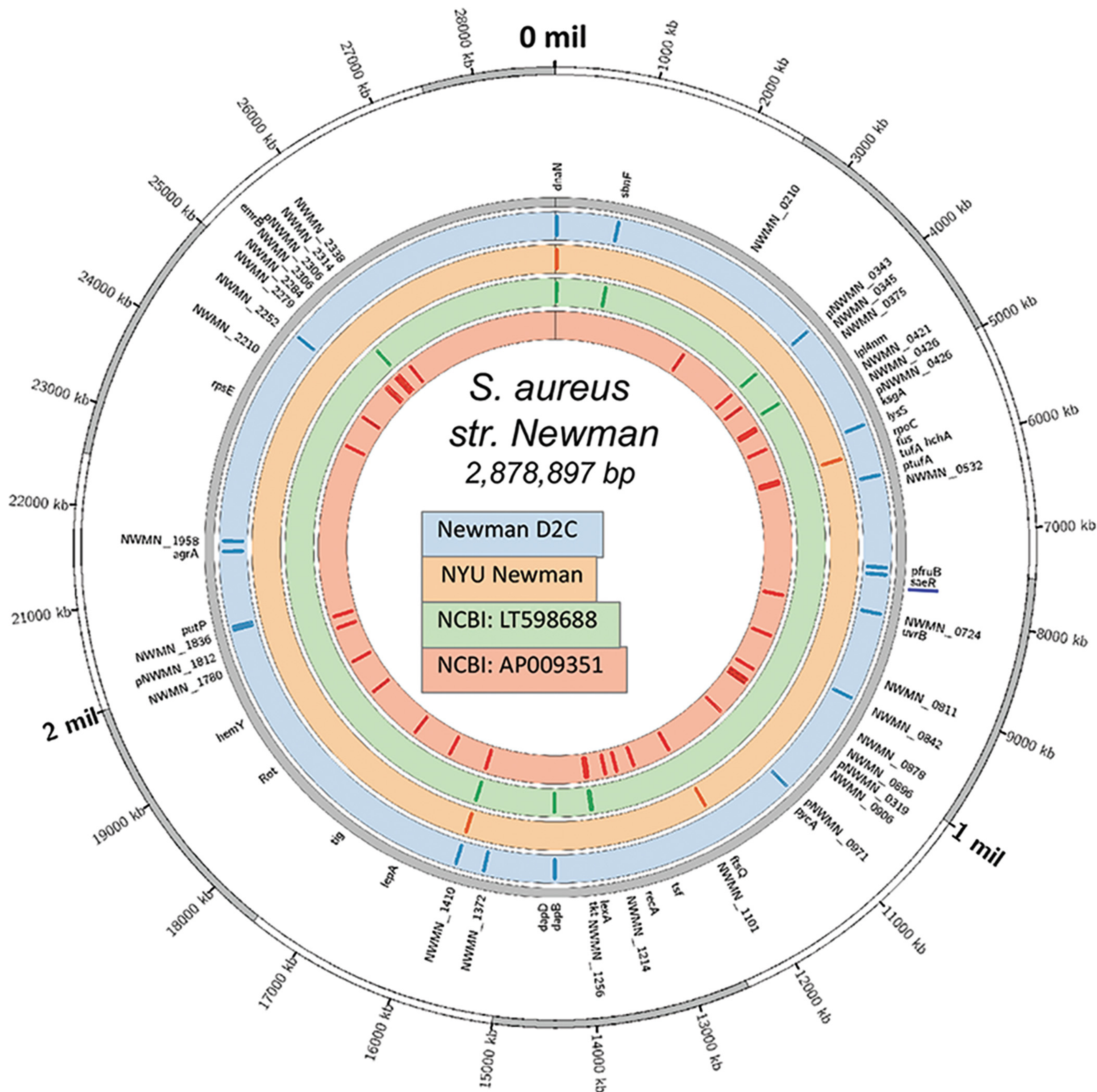

FIG 2 Whole-genome sequencing reveals disparities between Newman D2C and NYU Newman. Illumina HiSeq2000 sequencing demonstrated crucial mutations in Newman D2C compared to NYU Newman. Shown is a visualization of *S. aureus* Newman genetic diversity on a circular map of the chromosome of the Newman reference strain AP009351. The first ring from the outside shows the scale of the chromosome in nucleotides. The second ring indicates the positions of mutated genes in the genome (gray). The next four circles illustrate the four genomes used in this study (Newman D2C, NYU, NCBI LT598688, and NCBI AP009351). The colored tiles inside each circle represent the positions of mutations within each respective genome.

regulating *S. aureus* pathogenesis, thus likely accounting for the phenotypic abnormalities observed in the strain. Namely, we uncovered the presence of a stop codon in the ORF for the universal virulence gene activator AgrA (*agrA*), confirming the lack of toxin production described above, and a nonsynonymous SNP in the gene for the virulence gene regulator SaeR (*saeR*). These two regulatory proteins have been demonstrated to impact *S. aureus* pathogenesis (57–60), and thus, it is not surprising that Newman D2C displays a weak virulence phenotype in animal and *ex vivo* models of pathogenesis.

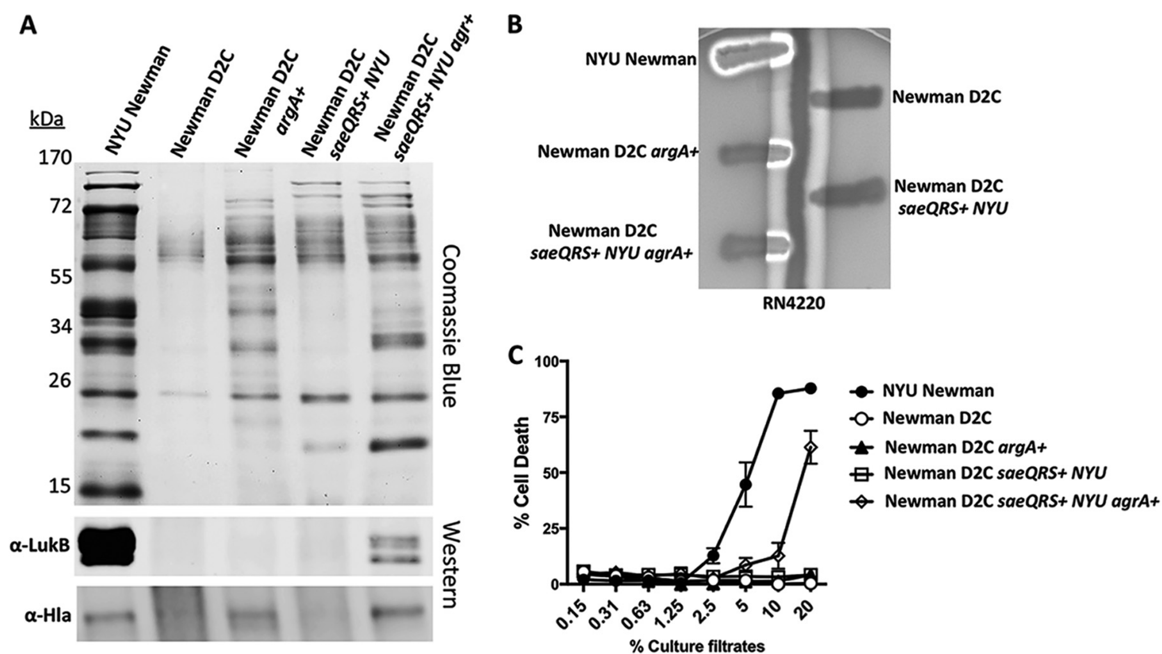

FIG 3 Addition of AgrA partially complements Newman D2C virulence deficiency. (A) Complementation of the Agr and Sae regulatory systems enhanced secretion in Newman D2C. (B) δ-Hemolysin activity was restored in Newman D2C upon the addition of AgrA. (C) Partial recovery of cytotoxicity was achieved in Newman D2C only in the presence of both functional Agr and Sae systems. The error bars indicate standard errors of the means.

Restoration of AgrA function does not reestablish virulence of Newman D2C. The most glaring mutation in the list of mutations that we uncovered in the Newman D2C strain was a stop codon in the ORF of the virulence gene regulator, *agrA*. The response regulator AgrA has been demonstrated to be critical for the production of a number of key *S. aureus* virulence factors (46, 53), including the transcription of the virulence gene activator RNAIII and the production of phenol-soluble modulins (PSMs) (54). As is evident in Fig. 1D, Newman D2C has a severe secretion deficiency compared to NYU Newman. To address this paucity of secretion, we wanted to determine if the addition of AgrA in *trans* could restore the strain's ability to secrete proteins. Upon the introduction of a wild-type *agrA* allele on a high-copy-number plasmid in Newman D2C, it became evident that the *agr* mutation accounted for only a fraction of the strain's deficiencies, as overexpression of *agrA* did not fully restore secretion in Newman D2C (Fig. 3A). In contrast, overexpression of *agrA* fully restored the production of δ-toxin by Newman D2C (Fig. 3B), a toxin controlled by AgrA (61), consistent with the functionality of the *agrA* overexpression plasmid (62).

We next sought to establish the less obvious role of the SNP occurring in the virulence gene regulator gene *saeR*. To make this assessment, we introduced the *saeQRS* locus from NYU Newman onto the chromosome at the SapI1 site of Newman D2C (63). We introduced the *saeQRS* locus, excluding *saeP* because subsequent complementation experiments utilized our previously published *saeQRS* mutant in the NYU Newman background; this mutant has no Sae function and can be complemented with *saeRS* in *trans* (64). The integration of *saeQRS* had a modest impact on the levels of protein production in Newman D2C (Fig. 3A). However, the tandem introduction of *agrA* and *saeQRS* with NYU Newman sequences bestowed the greatest enhancement in protein production for Newman D2C (Fig. 3A). Moreover, the synergism between AgrA and SaeR was highlighted by the production of specific *S. aureus* secreted virulence factors. Where Hla could be visualized upon the expression of functional *agrA*, expression of functional *agrA* and *saeQRS* resulted in greater production of the toxin. Furthermore, LukB and other leukocidins were detectable only when both response regulators were present in Newman D2C (Fig. 3A).

We next assessed the ability of the Newman D2C complement strains to induce hemolysis. Interestingly, the only detectable hemolytic activity that was restored was the function of δ-hemolysin in the presence of AgrA (Fig. 3B). This is a logical result, as it has been well documented that Agr contributes to the activation of this particular hemolysin (65). Of note, Newman D2C has a mutation in the dihydrodipicolinate reductase gene, *dapB*, a gene that lies adjacent to *dapA*, which has been previously shown to be crucial for hemolysis in Newman (66). Thus, this could explain the lack of full α-hemolysin activity despite the reconstitution of Agr and Sae.

Given Newman D2C's heightened level of protein secretion in the presence of functional AgrA and SaeR, we next wanted to determine whether this resulted in restoration of cytotoxicity. Again, supporting the synergism between these two regulators, human PMNs were susceptible to intoxication by culture filtrates from Newman D2C only when the strain was complemented with both NYU Newman *agrA* and *saeQRS* (Fig. 3C). However, this increase in cytotoxicity was still substantially lower than that of NYU Newman, indicating that the other mutations in Newman D2C are likely important for complete restoration of cytotoxicity.

The SNP in the Newman D2C *saeR* allele renders the SaeRS system nonfunctional. We next wanted to further dissect the significance of the SNP occurring in the ORF of the *saeR* allele (C595T, resulting in the amino acid change R199 to C199) in Newman D2C. While our complementation studies in Newman D2C (Fig. 3) certainly suggested that the NYU Newman *saeR* allele conferred greater virulence-activating power, we did not clarify the extent to which the Newman D2C *saeR* allele was attenuated. To address this question, we integrated copies of the *saeQRS* locus from either Newman D2C (Δ*saeQRS*::*saeQRS D2C*) or NYU Newman (Δ*saeQRS*::*saeQRS NYU*) into the SapI1 locus on the chromosome of a Δ*saeQRS* mutant in the NYU Newman background. Importantly for functionality studies, we first confirmed that both Δ*saeQRS*::*saeQRS NYU* and Δ*saeQRS*::*saeQRS D2C* were capable of producing SaeR protein (Fig. 4A). To assess the extent of attenuation in SaeR activity from Newman D2C, we first examined exoproteome profiles from the Δ*saeQRS* complemented strains. Interestingly, we found that while the *saeQRS NYU* allele was able to substantially restore protein secretion in the Δ*saeQRS* mutant, the *saeQRS D2C* allele provided no restoration of protein secretion (Fig. 4B). Moreover, upon evaluating toxin production, we witnessed a reconstitution of LukB and Hla production in Δ*saeQRS*::*saeQRS NYU* but not in Δ*saeQRS*::*saeQRS D2C* (Fig. 4B). While we did not see a recovery of hemolysis upon the integration of the *saeQRS NYU* allele into Newman D2C (Fig. 3B), we did, as expected, observe a reconstitution of hemolytic activity upon the integration of the *saeQRS NYU* allele into Δ*saeQRS* (Fig. 4C). The *saeQRS D2C* allele was unable to restore hemolysis, further highlighting the weakened activity of the system in the Newman D2C strain.

The Newman D2C *saeR* allele confers no pathogenic activity. To assess whether the inability to produce secreted proteins at wild-type levels impacted pathogenesis, we determined whether Δ*saeQRS*::*saeQRS D2C* could elicit cytotoxic properties. Further substantiating the observation that the Newman D2C SaeR protein could not activate the expression of secreted proteins, we found that only the *saeQRS NYU* allele was capable of restoring cytotoxicity in the Δ*saeQRS* mutant (Fig. 5A). Our *in vitro* assessment of protein production and *ex vivo* assays with human PMNs suggested that the *saeR* allele in Newman D2C produces an inactive protein. To verify this notion, we tested the pathogenicity of the Δ*saeQRS* complement strains in a mouse model of bacteremia. We observed that the *saeQRS NYU* allele restored lethality to the *saeQRS* mutant while Δ*saeQRS*::*saeQRS D2C* behaved very similarly to the *saeQRS*-null mutant in that it was essentially nonpathogenic (Fig. 5B). While statistically insignificant, the slightly increased lethality observed in the Δ*saeQRS*::*saeQRS NYU* strain compared to that of wild-type NYU Newman was likely due to the placement of the *saeQRS* at the Sap1I site (67). Further confirming the lack of functionality of the Newman D2C *saeQRS* allele, we found that the bacterial burden in critical organs was significantly reduced in

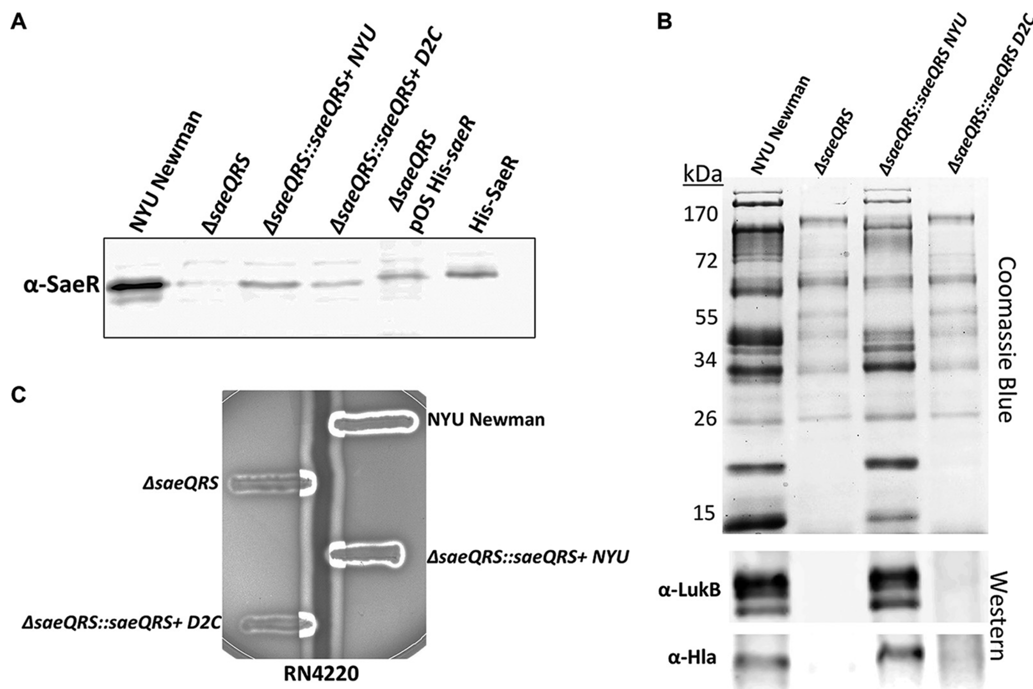

FIG 4 A unique SNP in *saeR* from Newman D2C renders the protein product nonfunctional. (A) Western blot demonstrating that a Δ*saeQRS* mutant complemented with *saeQRS* from either Newman D2C or NYU Newman produced SaeR protein. SaeR produced from a plasmid and purified SaeR served as positive controls; these proteins carried a histidine tag. (B) Only the *saeQRS* allele from NYU Newman was capable of restoring protein secretion in a complemented Δ*saeQRS* mutant. (C) The Newman D2C *saeQRS* allele could not reconstitute hemolysis in a Δ*saeQRS* mutant, while the NYU Newman *saeQRS* allele could restore hemolysis.

mice that had been infected with the Δ*saeQRS*::*saeQRS D2C* strain compared to those infected with the Δ*saeQRS*::*saeQRS NYU* strain (Fig. 5C). While the *saeQRS*-null mutant complemented with the *saeQRS NYU* allele was able to regain wild-type levels of bacterial burden in the kidney, full restoration of the bacterial burden was not achieved in the heart (Fig. 5C). This is again likely due to the expression dynamic of placing the *saeQRS* locus at the Sap1 site on the chromosome.

DISCUSSION

In order to develop an effective therapy to combat the deadly consequences of invasive *S. aureus* infection, models that appropriately demonstrate the bacterium's pathogenic potential must be utilized. Here, we reveal that the commonly used *S. aureus* strain Newman D2C from the ATCC, used in multiple studies of antistaphylococcal therapeutics and vaccines (25–30), contains numerous mutations that render the strain essentially nonpathogenic. Remarkably, Newman D2C contains both a stop mutation in the virulence gene activator gene, *agrA*, and a novel polymorphism in the virulence gene regulator gene, *saeR*, that abolishes SaeR function. The importance of these two mutations is highlighted by the partial recovery of virulence that we observed in Newman D2C upon complementation of both of these pathways. Additionally, it is clearly evident that the remaining mutations in Newman D2C are significant, as we were unable to achieve full restoration of virulence with both functional Agr and Sae systems. Complete analysis of the remaining mutations in Newman D2C (see Table S1 in the supplemental material) would be required to fully understand the extent of Newman D2C's pathogenic shortcomings.

Here, we show that a previously unidentified polymorphism occurring in *saeR* eliminates the functionality of the protein product. This loss of function can likely be attributed to both the location of the SNP on the chromosome and the nature of the amino acid alteration that occurs. Specifically, amino acid 199 in SaeR from NYU

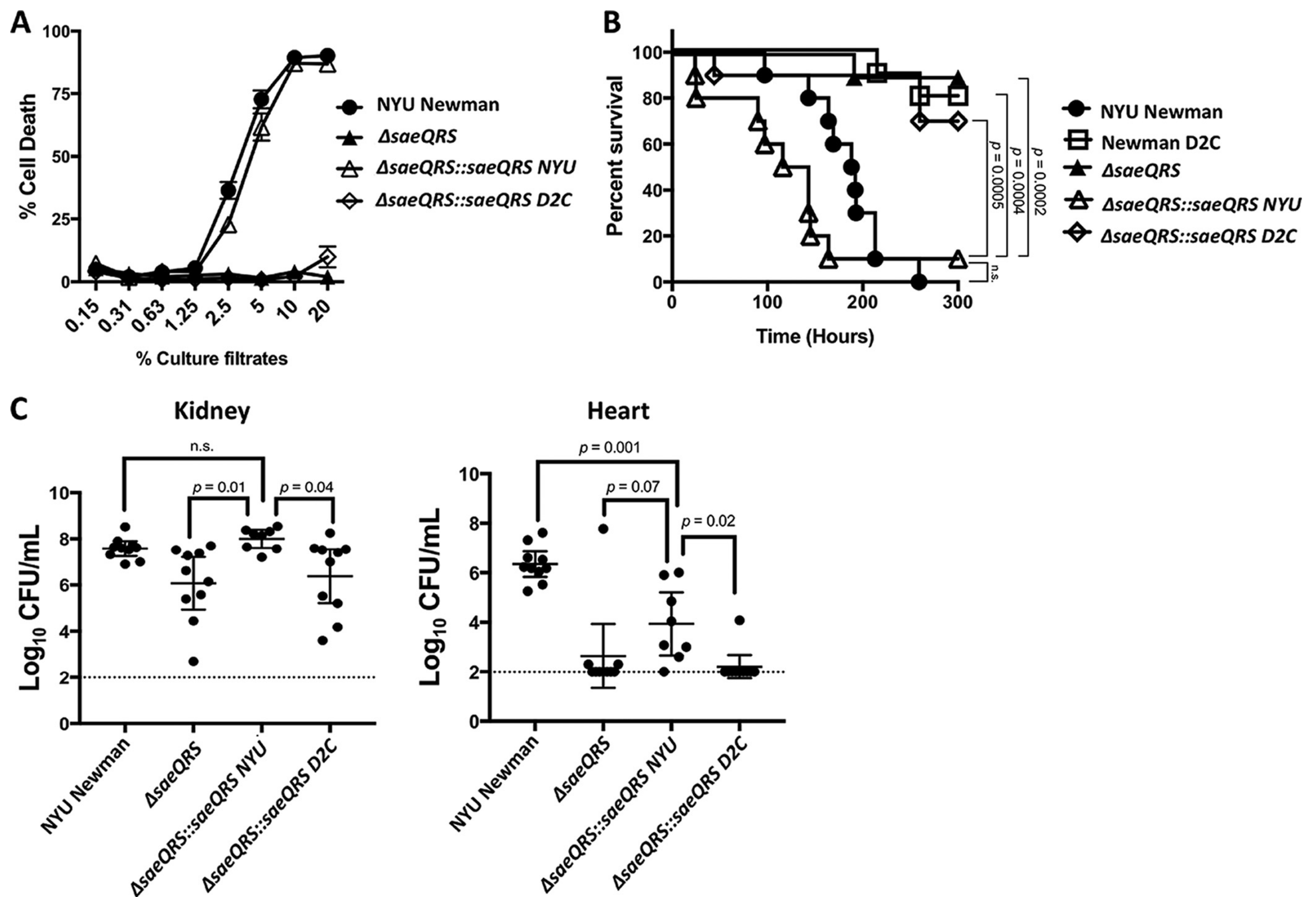

FIG 5 SNP C595T in Newman D2C abolishes pathogenesis. (A) The NYU Newman *saeR* allele restored cytotoxicity to an Δ*saeQRS* mutant, whereas the *saeQRS* allele from Newman D2C provided no restoration of cytotoxicity ($n = 6$ donors). Intoxications were repeated with unique transductants for both Δ*saeQRS*::*saeQRS D2C* and Δ*saeQRS*::*saeQRS NYU*, and the same results were achieved (data not shown). (B) Mice infected intravenously were susceptible to acute lethality only in the presence of the NYU Newman *saeQRS* allele, as neither Newman D2C, Δ*saeQRS*, nor Δ*saeQRS*::*saeQRS D2C* had acutely lethal effects on the mice ($n = 10$ mice/strain). The points indicate individual mouse deaths. Infections were done in sets of 5 mice, and each infection was performed with a unique transductant for Δ*saeQRS*::*saeQRS D2C* and Δ*saeQRS*::*saeQRS NYU*. Statistical analysis for survival was performed using the Gehan-Breslow-Wilcoxon test with *P* values adjusted for multiple comparisons. (C) Mice infected intravenously exhibited reduced bacterial burdens in critical organs when infected with either Δ*saeQRS* or Δ*saeQRS*::*saeQRS D2C* compared to that of NYU Newman or Δ*saeQRS*::*saeQRS NYU* ($n = 10$ mice/strain). Dotted lines represent the limit of detection for bacterial burden in these organs. Statistical analysis was performed using one-way analysis of variance (ANOVA) with *post hoc* Holm-Sidak multiple-comparison tests where appropriate. The error bars indicate standard errors of the means.

Newman is arginine (R199), while in Newman D2C, the arginine has been replaced with cysteine. The mutation is significant because this residue in SaeR has been shown to be a crucial residue in the protein's DNA binding domain (68). In an effort to identify the DNA binding domain of SaeR, Fan et al. demonstrated that upon replacing the arginine 199 residue with alanine, *S. aureus* loses the ability to produce alpha toxin and to induce hemolysis (68). Moreover, purified SaeR containing the R→A199 substitution exhibited reduced binding to the P1 promoter region of the *sae* operon, while wild-type SaeR maintained its high binding affinity (68). Thus, the loss of function in SaeR from Newman D2C can likely be attributed to the protein product's inability to bind critical promoter regions associated with virulence gene expression.

This study also emphasizes the importance of scrupulously examining genomic-sequence data when assessing bacterial virulence. Here, we show that strain Newman D2C from the ATCC, a strain that has been used in numerous studies that sought to assess *S. aureus* pathogenesis in both *in vivo* and *ex vivo* models of infection (25–30), is in fact nonpathogenic. The chain of custody of strain Newman D2C at the ATCC is listed as E. Duthie (1952) to J. Hawiger (1970) to the ATCC, and it was originally described as a clumping-factor-positive variant of Newman strain D2 (23), although the relationship

between the original Newman strain and the Newman D2 strain is not apparent. Unfortunately, Newman D2C has been the centerpiece of multiple studies on *S. aureus* pathogenesis and antistaphylococcal therapeutics and vaccines that have substantially influenced the field's understanding of both *S. aureus* biology and assessments of the efficacy of antistaphylococcal agents (25–30) and is often referred to simply as strain Newman. It should be noted that the references listed here are by no means a comprehensive list of all the publications that have used Newman D2C as their model organism, as that would be quite extensive. While the conclusions from the majority of studies using Newman D2C may be sound, the data described here bring the results of some studies into question. This is particularly problematic for studies that have assessed the efficacy of therapeutic agents, as the threshold for positive data is likely lowered with models using Newman D2C. While it may be impractical to continually sequence bacterial stocks of commonly used strains, a range of basic tests are available for *S. aureus* to evaluate the integrity of the bacterium's virulence. Some of these assessments are described here, including examining for hemolysis, profiling of protein secretions, and determining *ex vivo* cytotoxicity. As efforts continue to identify and characterize novel antibacterial agents and vaccines, these studies provide a cautionary tale in ensuring that the integrity of the virulence of the model organisms employed is maintained and is consistent between laboratories.

MATERIALS AND METHODS

Bacterial culture conditions. The *S. aureus* strains used in this study were grown at 37°C on tryptic soy agar (TSA) or broth (TSB). When appropriate, the strains were grown in the presence of either tetracycline (4 μg/ml) or chloramphenicol (10 μg/ml). *Escherichia coli* DH5α was used for cloning and propagation of plasmids. *E. coli* culturing was done in Luria-Bertani broth supplemented with ampicillin (100 μg/ml). Liquid cultures were grown in 5 ml of growth medium in 15-ml conical tubes incubated at a 45° angle with shaking at 180 rpm. For all experiments involving the growth of *S. aureus* bacteria, overnight cultures were diluted 1:100 in fresh TSB.

Genome sequencing, assembly, and annotation. We prepared sequencing libraries from DNA extracted from Newman D2C and NYU Newman as previously described (69). Whole-genome sequencing was performed using an Illumina HiSeq2000 with 100-base paired-end reads. The paired-end Illumina reads were mapped against the *S. aureus* Newman reference genomes AP009351.1 and LT598688 using the Burrows-Wheeler Aligner (BWA) (70). AP009351.1 was described by Baba et al. and is the parental strain of NYU Newman (22). The LT598688 sequence was deposited by Monk et al. (49). The BWA outputs were analyzed and annotated using SAMtools (71), GATK (72), and ANNOVAR (73). SNPs in genes annotated as integrases, transposases, resolvases, maturases, or phages were removed from the analysis. Other mobile genetic elements, including SaPI5, phiSA2usa, phiSA3usa, SCCmecIV, and ACME, were identified using IslandPath-DIMOB and PHASTSNPs (74, 75). Genomes and mutations were visualized using Circos (76).

Construction of bacterial strains. All the strains and plasmids used in this study are listed in Tables 1 and 2. Complementation of *saeQRS NYU* or *saeQRS D2C* in the chromosome of Δ*saeQRS* or Newman D2C was performed with the pJC1306 suicide plasmid (63), which stably integrates DNA into the SaPI1 site, leading to a single-copy chromosomal insertion. The only difference between the NYU Newman *saeQRS* locus and the Newman D2C *saeQRS* locus occurs at the C595T polymorphism in *saeR*, and as such, the same primer set was used to amplify the *saeQRS* loci from both strains. Primers VJT1796 (5′ CCCCCCCCTGCAGTCAATTTCTGAGTTAAACT 3′) and VJT1797 (5′ CCCCCCCGGATCCTTATGACGTAATGTCT AATT 3′) amplified a product that extends from the 5′ intergenic region upstream of *saeQ* to the 3′ end of the *saeS* ORF. Within this genetic fragment, a transcriptional promoter exists in *saeQ*'s intragenic region (39). These products were then digested with BamHI and PstI, cloned into pJC1306, and transformed into *E. coli* DH5α. Sanger sequencing was used to verify that the C595T polymorphism in *saeR* was present in the pJC1306/*saeQRS D2C* plasmid, and wild-type *saeR* was similarly confirmed in pJC1306/*saeQRS NYU*. To generate the integrated strains, we first transformed pJC1306 *saeQRS* (*NYU* or *D2C*) or pJC1306 (empty vector) plasmids into RN4220 carrying pRN7023, which contains an integrase and thus allows integration of pJC1306 into the SaPI1 site (63). The SapI1 locus from RN4220 was then transduced with phage Φ80 into either Δ*saeQRS NYU* Newman, Newman D2C, or NYU Newman. Strains harboring the plasmid pOS*plgt-agrA* were transformed via electroporation.

Exoprotein analysis. Proteins were concentrated from 5-h culture supernatants using 10% (vol/vol) trichloroacetic acid (TCA) precipitation as described previously (46). Protein visualization was achieved by separating samples using SDS-12% PAGE and staining with Coomassie brilliant blue. To assess the production of specific products, proteins were resolved with 12% SDS-PAGE, transferred to a nitrocellulose membrane, and then probed with the indicated primary antibody. Alex Fluor 680–anti-rabbit antibody was used as a secondary antibody, and the membranes were visualized using the Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE).

Cell lysate preparation. Five-hour *S. aureus* subcultures were centrifuged, and the cell pellets were washed one time in 1× PBS. Following the wash, the pellets were resuspended in 1 ml of TSM (50 mM

TABLE 1 Bacterial strains used in this study

Strain	Background	Description	Designation	Reference
VJT 3.81	NYU Newman	Wild-type NYU Newman	NYU Newman	21
VJT 50.23	Newman D2C	Newman D2C (10833)	Newman D2C	21, 23
VJT 57.16	NYU Newman	NYU Newman carrying the empty pJC1306 vector at the SapI1 site	NYU Newman	This study
VJT 57.15	Newman D2C	Newman D2C carrying the empty pJC1306 vector at the SapI1 site	Newman D2C	This study
VJT 2.60	RN4220	Wild-type RN4220	RN4220	77
VJT 3.76	NYU Newman	Newman carrying the *bursa aurealis* transposon in the *hla* gene	*hla*::*bursa*	78
VJT 8.91	NYU Newman	Newman with a clean deletion of the *lukAB* locus	Δ*lukAB*	8
VJT 57.17	NYU Newman	Δ*saeQRS*::*spec* carrying the empty pJC1306 vector at the SapI1 site	Δ*saeQRS*	This study
VJT 57.10	NYU Newman	Δ*saeQRS*::*spec* carrying pJC1306 expressing *saeQRS NYU* in the SaPI1 site	Δ*saeQRS*::*saeQRS NYU*	This study
VJT 57.09	NYU Newman	Δ*saeQRS*::*spec* carrying pJC1306 expressing *saeQRS D2C* in the SaPI1 site	Δ*saeQRS*::*saeQRS D2C*	This study
VJT 50.43	NYU Newman	NYU Newman carrying the empty pOS1p*lgt* vector	NYU Newman	This study
VJT 50.45	Newman D2C	Newman D2C carrying the empty pOS1p*lgt* vector	Newman D2C	This study
VJT 50.48	Newman D2C	Newman D2C carrying the pOS1p*lgt agrA* vector	Newman D2C *argA*$^{+}$	This study
VJT 57.32	Newman D2C	Newman D2C carrying the empty pOS1p*lgt* vector and pJC1306 expressing *saeQRS NYU* in the SaPI1 site	Newman D2C::*saeQRS NYU*	This study
VJT 57.33	Newman D2C	Newman D2C carrying the pOS1p*lgt agrA* vector and pJC1306 expressing *saeQRS NYU* in the SaPI1 site	NewmanD2C::*saeQRS NYU agrA*$^{+}$	This study
VJT 31.57	NYU Newman	Δ*lukED:hlgACB*::*tet*::*lukAB*::*spec*::*hla*::*ermC*	ΔΔΔΔ	11
VJT 16.99	NYU Newman	Δ*saeQRS*::*spec*	Δ*saeQRS*	64

Tris, 0.5M D-sucrose, 10 mM $MgCl_2$, pH 7.5) and 10 μl of 2-mg/ml lysostaphin and then incubated at 37°C for 10 min. Following incubation, samples were centrifuged and the pellets were resuspended in 250 μl of 1× PBS. The samples were then moved to tubes containing 0.1-mm glass beads and disrupted using an MP Biomedicals FastPrep-24 homogenizer. The resulting samples were then centrifuged, and the soluble fraction was resuspended in 2× SDS sample buffer.

Ethics statement. Buffy coats were obtained from anonymous blood donors with informed consent from the New York Blood Center. Because all of the samples were collected anonymously prior to their delivery, the New York University Langone Medical Center (NYULMC) Institutional Review Board determined that our study was exempt from further ethics approval requirements.

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of NYULMC. All experiments were performed according to NIH guidelines, the Animal Welfare Act, and U.S. federal law.

Cytotoxicity assays. To evaluate the cytotoxicity of the secreted factors produced from each strain, primary human PMNs were intoxicated with culture filtrates obtained from 5-h *S. aureus* subcultures. Prior to intoxication, culture filtrates were diluted 2-fold in a 96-well plate (20 to 0.15%). PMNs were isolated as described by Reyes-Robles et al. (11) and normalized to 200,000 cells per 80 μl RPMI (10 mM HEPES plus 0.1% human serum albumin). PMNs (80 μl) were then pipetted into each well, and the supernatant-PMN mixtures were incubated in a 37°C-5% CO_2 incubator for 1 h. To assess toxicity, 10 μl of CellTiter 96 Aqueous One solution (CellTiter; Promega) was added to the 96-well plate, and the mixture was incubated at 37°C in 5% CO_2 for 1.5 h. PMN viability was assessed with a PerkinElmer EnVision 2103 multilabel reader at an absorbance of 492 nm.

Murine sepsis model. Three-hour subcultures of *S. aureus* were washed, resuspended in 1× phosphate-buffered saline, measured for cell density (optical density at 600 nm [OD_{600}]), normalized, and then plated for CFU. The initial experiment comparing tissue burdens in mice infected with NYU Newman and Newman D2C (purchased by and obtained from Janssen R&D LLC) used 5 × 10^7 CFU; the remaining tissue colonization experiments were conducted with 2.5 × 10^7 CFU. All mouse survival infections were conducted with 5 × 10^7 CFU. Retro-orbital infections (100-μl inoculum) were performed on 5-week-old

TABLE 2 Plasmids used in this study

Name	Description	Resistance[a]	Reference
pOS1-P*lgt*	*lgt* promoter in an empty vector	Cm	79
pOS1-P*lgt-agrA*	*lgt* promoter driving *agr* expression	Cm	62
pJC1306	Single-copy integration vector that inserts at the SaPI1 site	Tet	63
pJC1306/*saeQRS NYU*	Plasmid pJC1306 expressing *saeQRS NYU* from its native promoter	Tet	This study
pJC1306/*saeQRS D2C*	Plasmid pJC1306 expressing *saeQRS D2C* from its native promoter	Tet	This study
pOS1-P*lgt-saeR*	*lgt* promoter driving *saeR* expression	Cm	This study

[a]Cm, chloramphenicol; Tet, tetracycline.

female ND4 Swiss-Webster mice (Envigo) that had been anesthetized intraperitoneally with 300 μl of Avertin (2,2,2-tribromoethanol dissolved in *tert*-amyl alcohol and diluted to a final concentration of 2.5% [vol/vol] in sterile saline). To examine for tissue colonization at 96 h postinfection, the mice were euthanized with CO_2, and the indicated organs were harvested as described previously (9). For acute/survival experiments, mice administered retro-orbital infections were monitored every 4 to 6 h for signs of morbidity (hunched posture, lack of movement, paralysis, and inability to acquire food or water), at which time the animals were euthanized and survival curves were plotted.

Accession number(s). All the genomic data are available at NCBI under the following accession numbers: NYU Newman, CP023390.1; ATCC Newman, CP023391.1.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00476-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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V.J.T. is an inventor on patents and patent applications filed by New York University, which are currently under commercial license to Janssen Biotech Inc. B.J.M., P.T.B., J.F., and A.S.L. are employees of Janssen Research and Development and declare competing financial interests.

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