NIH Public Access

Author Manuscript

Mol Microbiol. Author manuscript; available in PMC 2013 January 1.

Published in final edited form as:

Mol Microbiol. 2012 January ; 83(2): 423–435. doi:10.1111/j.1365-2958.2011.07942.x.

Staphylococcus aureus leukocidin ED contributes to systemic infection by targeting neutrophils and promoting bacterial growth *in vivo*

Francis Alonzo III[1], **Meredith A. Benson**[1], **John Chen**[3], **Richard P. Novick**[1,3], **Bo Shopsin**[1,2], and **Victor J. Torres**[1]

[1]Department of Microbiology, New York, New York 10016, USA

[2]Department of Medicine, New York, New York 10016, USA

[3]Skirball Institute of Biomolecular Medicine New York University School of Medicine, New York, New York 10016, USA

SUMMARY

Bloodstream infection with *Staphylococcus aureus* is common and can be fatal. However, virulence factors that contribute to lethality in *S. aureus* bloodstream infection are poorly defined. We discovered that LukED, a commonly overlooked leukotoxin, is critical for *S. aureus* bloodstream infection in mice. We also determined that LukED promotes *S. aureus* replication *in vivo* by directly killing phagocytes recruited to sites of hematogenously-seeded tissue. Furthermore, we established that murine neutrophils are the primary target of LukED, as the greater virulence of wild type *S. aureus* compared to a *lukED* mutant was abrogated by depleting neutrophils. The *in vivo* toxicity of LukED toward murine phagocytes is unique among *S. aureus* leukotoxins, implying its crucial role in pathogenesis. Moreover, the tropism of LukED for murine phagocytes highlights the utility of murine models to study LukED pathobiology, including development and testing of strategies to inhibit toxin activity and control bacterial infection.

Keywords

Staphylococcus aureus; MRSA; bi-component leukotoxin; neutrophil; bacteremia; LukED

Introduction

S. aureus bacteremia, coupled with subsequent dissemination to and damage of distant tissue sites, is responsible for significant morbidity and mortality in the United States and throughout the world (Klevens *et al.*, 2006, Klevens *et al.*, 2007). A hallmark of highly virulent *S. aureus* strains is their ability to effectively kill neutrophils (Voyich *et al.*, 2005), key innate immune effector cells absolutely required for control of infection (Pincus *et al.*, 1976, Lekstrom-Himes & Gallin, 2000, Dale *et al.*, 1979, Bodey *et al.*, 1966). *S. aureus* uses multifaceted and often redundant mechanisms to ensure broad protection against attack by host neutrophils (Foster, 2005, Nizet, 2007).

One major mechanism by which *S. aureus* targets and kills neutrophils *in vitro* is through the production of bi-component pore-forming leukotoxins (Menestrina *et al.*, 2003). Strains

Corresponding Author: Victor J. Torres, Ph.D., 522 First Ave. SRB 1010, New York City, NY 10016, Phone: (212)-263-9232, Fax: (212)-263-9180, victor.torres@nyumc.org.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

associated with human infections can produce up to four different bi-component leukotoxins: γ-hemolysin (HlgACB), LukSF-PVL, LukAB/HG, and LukED. Among these toxins, HlgACB is believed to play a role in septic arthritis and weight loss upon systemic infection (Nilsson *et al.*, 1999), contribute in part to the inflammatory response observed in the rabbit eye *in vivo* (Supersac *et al.*, 1998), as well as contribute modestly to community-acquired methicillin resistant *S. aureus* (CA-MRSA) survival in human blood and virulence upon systemic infection of mice (Malachowa *et al.*, 2011). Studies of the contribution of PVL to *S. aureus* pathogenesis on the other hand have led to conflicting conclusions due in part to the toxin's species specificity, but PVL is believed to contribute to pneumonia (Labandeira-Rey *et al.*, 2007, Voyich *et al.*, 2006, Loffler *et al.*, 2010, Diep *et al.*, 2010). Recently LukAB/HG, a new member of the *S. aureus* leukotoxin family, was shown to contribute to neutrophil killing; promote survival of *S. aureus* in human whole blood; restrict neutrophil-mediated killing; and promote CA-MRSA pathogenesis (Dumont *et al.*, 2011, Ventura *et al.*, 2010). Among the leukotoxins, LukED is the least characterized. LukED exhibits toxicity toward PMNs *in vitro* and induces dermonecrosis when purified toxin is injected into rabbits (Gravet *et al.*, 1998, Morinaga *et al.*, 2003). Despite all the effort devoted to the study of *S. aureus* leukotoxins, the direct mechanism of action of these toxins during the course of infection has not been defined.

In this work, we identify LukED as a major virulence factor involved in bloodstream infection with *S. aureus*. Our studies demonstrate for the first time that LukED plays a critical role in *S. aureus* lethality for mice. In stark contrast to other staphylococcal leukotoxins, we observed that LukED effectively targets and kills murine phagocytes, including neutrophils *ex vivo*. Investigation into the *in vivo* mechanism of action of LukED demonstrated that the toxin promotes disease progression via its potent cytotoxic effects on phagocytes recruited to hematogenously-seeded infection sites. These results underscore the potential role of LukED as a critical virulence factor required for bloodstream infection with *S. aureus*, including highly pathogenic MRSA.

Results

Using global regulators to dissect the contribution of secreted factors to S. aureus *systemic infection*

In an effort to identify individual virulence factors involved in bacteremia, we first investigated the contribution of the accessory gene regulatory (Agr) system to the lethality observed upon *S. aureus* systemic infection. The Agr system regulates the differential expression of *S. aureus* secreted and surface proteins in a quorum dependent manner (Novick & Geisinger, 2008). Mice were infected systemically with *S. aureus* Newman, a highly virulent clinical methicillin sensitive *S. aureus* (MSSA) strain (Duthie & Lorenz, 1952), as well as an isogenic Δ*agr* mutant lacking the entire *agr* locus. We observed that the Δ*agr* mutant was significantly attenuated for virulence compared to animals infected with wild type, as the animals did not succumb to infection (Fig. 1A).

The regulation of a number of important virulence factors (including *S. aureus* cytotoxins) by the Agr system is mediated in an RNAIII-dependent manner. RNAIII is a regulatory RNA molecule, expressed upon Agr activation, that influences translation of target mRNAs (Novick *et al.*, 1993, Novick & Geisinger, 2008). A major mechanism by which RNAIII modulates virulence factor expression is via its regulatory control over the transcription factor Rot (Repressor of toxins) (McNamara *et al.*, 2000, Geisinger *et al.*, 2006, Boisset *et al.*, 2007, Said-Salim *et al.*, 2003). RNAIII blocks translation of Rot by binding to *rot* mRNA (Geisinger et al., 2006, Boisset et al., 2007). The inhibitory binding of RNAIII to *rot* mRNA facilitates optimal expression of otherwise Rot-repressed cytotoxins (Said-Salim et al., 2003). To test whether the virulence defect of the Δ*agr* mutant is dependent on Rot-

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

regulated factors, we infected mice with an Δ*agr*Δ*rot* double mutant strain. We observed that the deletion of *rot* in the Δ*agr* strain fully restored virulence in mice (Fig. 1A), consistent with a previous report using a rabbit endocarditis model (McNamara & Bayer, 2005). To directly probe the contribution of Rot to *S. aureus* pathogenesis, a Δ*rot* mutant with a normal functioning *agr* locus was tested in the systemic infection model. The Newman Δ*rot* mutant strain was found to be hypervirulent compared to wild type (Fig. 1B). Taken together these results suggest that the regulatory input of Agr upon Rot directly influences bloodstream infection with *S. aureus*.

LukED is critical for the virulence of a Δrot *mutant*

Virulence factors responsible for death due to *S. aureus* bloodstream infection are poorly defined. The enhanced virulence of the Δ*rot* mutant suggests that a Rot-repressed factor contributes to lethality in *S. aureus* bloodstream infection. A major group of Rot-repressed factors are cytotoxin-encoding genes (McNamara et al., 2000, Said-Salim et al., 2003). To gain insight into the cytotoxin(s) potentially responsible for the enhanced virulence of Δ*rot* and the Δ*agr*Δ*rot* double mutant strains, we monitored cytotoxin abundance in culture supernatants via immunoblotting. We observed that toxin levels were markedly reduced in the Δ*agr* mutant compared to the wild type strain (Fig. 1C), a phenotype rescued by deleting *rot* in the Δ*agr* strain (Fig. 1C). In contrast to other toxins, the LukE subunit of the bi-component leukotoxin LukED was strikingly overproduced by the Δ*agr*Δ*rot* mutant (Fig. 1C). Similarly, we observed that the Δ*rot* strain produced increased amounts of LukE, while no major difference was observed for the other cytotoxins (Fig. 1D).

The increased production of LukE in the absence of Rot and the associated hypervirulence of a Δ*rot* mutant led us to hypothesize that LukED was involved in the increased virulence of the Δ*rot* strain. To directly test this hypothesis we constructed double mutants lacking both *rot* and each of the four major leukotoxin genes/operons present in strain Newman (*hla, hlgACB, lukAB/HG,* and *lukED*) (Dumont et al., 2011), and challenged mice systemically with each strain. We observed that deletion of *lukAB/HG*, and *hlgACB* caused modest but statistically significant reductions in the Δ*rot* hypervirulent phenotype (Fig. 2A-C) consistent with previously published roles for these toxins in the pathogenesis of *S. aureus* (Dumont et al., 2011, Nilsson et al., 1999, Malachowa et al., 2011). In stark contrast, a Δ*rot*Δ*lukED* double mutant was markedly reduced for virulence (Fig. 2D), suggesting that *lukED* plays a critical role in the hypervirulence exhibited by the Newman Δ*rot* mutant.

LukED promotes disease progression during systemic infection

To evaluate whether LukED directly contributes to lethality in *S. aureus* bloodstream infection, we constructed a Δ*lukED* mutant in strain Newman, as well as a complementation strain wherein *lukED* and its native promoter sequence were ectopically integrated into the chromosome (Fig. 3A). To verify that the Δ*lukED* mutant was altered only in LukED production, the toxin profile was analyzed by immunoblotting. We observed that only LukE production was altered by the Δ*lukED* mutant, a phenotype fully complemented in the Δ*lukED*::*lukED* strain (Fig. 3B). The strains were then used to challenge mice systemically as described above. These experiments revealed that the Δ*lukED* mutant was markedly attenuated for virulence compared to wild type, a phenotype completely restored in the complement strain (Fig. 3C).

LukED is critical for the pathogenesis of USA500 MRSA strains

To evaluate the contribution of LukED to the virulence of modern strains, we first determined whether the major clones of MRSA currently causing infections in the United States, pulse field electrophoresis types USA100, USA200, USA300, USA400, and USA500 (Klevens et al., 2007), contained the *lukE/D* genes. All strain types, excluding USA200,

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

contained the *lukE/D* genes (Table 1) (Diep *et al.*, 2006). Additionally all *lukED*-containing strains were capable of expressing *lukE* mRNA as determined by qRT-PCR (Fig. S1A). Among the *lukED* positive strains, USA300, USA400, and USA500 are considered to be the most virulent in animal models (Li *et al.*, 2010, Li *et al.*, 2009). We observed that *S. aureus* USA500 and Newman were far more virulent in mice systemically infected with 1×10^7 CFU compared to USA300 and USA400 strains (Fig. S1B). USA500 strains are associated with both hospital and community acquired infections (Diep et al., 2006, Klevens et al., 2007, Li et al., 2009). However, virulence factors involved in the pathogenesis of USA500 are poorly defined. To evaluate the contribution of *lukED* to USA500 infection, we constructed Δ*lukED* mutants in two independent USA500 clinical isolates (i.e. BK2371 and BK2395) and subsequently tested their virulence potential. Deletion of *lukED* markedly attenuated the virulence potential of both strains (Fig. 3D), suggesting that LukED is a major determinant of USA500 virulence.

LukED facilitates bacterial replication in vivo

To determine the specific contribution of LukED to hematogenous infection, we monitored colonization, bacterial replication, and abscess formation in the kidneys of animals infected systemically with *S. aureus*. Compared to Newman wild type or the Δ*lukED*::*lukED* complemented strain, a Δ*lukED* mutant exhibited significantly reduced abscess formation after 96-hours (Fig. 4A). We reasoned that a reduction in abscess formation could indicate either (i) an inability of the Δ*lukED* mutant to seed the kidney of infected animals or (ii) an inability of the Δ*lukED* mutant to replicate in seeded kidneys due to better control of infection by immune cells. To test both possibilities, we monitored bacterial burden to the kidney at 16 and 96 hours (Fig. 4B). Total CFU in the kidneys early after infection were identical for all strains. In contrast, at 96 hours a Δ*lukED* mutant exhibited a 15 fold decrease in bacterial burden compared to both wild type and the complemented strain. Consistent with the observed differences in bacterial burden, animals infected with the Δ*lukED* mutant also exhibited reduced markers of inflammation (IL-6 and GCSF) in the serum at 96 hours (Fig. 4C). Collectively, these findings suggest that LukED contributes to the virulence of *S. aureus* by promoting bacterial proliferation within hematogenously-seeded tissue.

LukED targets and kills neutrophils by damaging their plasma membrane

One mechanism by which LukED could promote *S. aureus* virulence is through the killing of neutrophils. To determine whether LukED is cytotoxic towards primary murine neutrophils, we isolated peritoneal elicited cells (PECs) from animals infected with *S. aureus*. Infection of the peritoneum induced a robust infiltration of neutrophils ($CD11b^+$/$Ly6G^+$, ~66%) (Fig. 5A-B). Isolated PECs were intoxicated with purified recombinant LukE, LukD, or an equimolar mixture of LukE and LukD (LukED). Intoxication with high doses of any single toxin subunit (10μg/ml) exhibited negligible cytotoxic effects towards PECs (Fig. 5A-B). In contrast, intoxication with both subunits significantly reduced the number of viable PECs from ~50% to ~15% (Fig. 5A). Within the PEC population neutrophils were specifically targeted, as over 85% of $Ly6G^+$/$CD11b^+$ cells were killed (Fig. 5B).

LukED intoxicated PECs, but not PECs intoxicated with single subunits, exhibited characteristic morphological alterations associated with membrane permeabilization and cell death (nuclei swelling, cell rounding, and membrane halos) (Fig. 6A). Other indicators of rapid cell death included a dose dependent decrease in metabolic activity as measured via CellTiter (Fig. 6B), and overt membrane destabilization as determined via lactate dehydrogenase release into culture medium within one hour of intoxication (Fig. 6C). Additionally, ethidium bromide uptake, an assay typically used as an indicator of pore-

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

formation (Finck-Barbancon *et al.*, 1993), was observed as early as 15 minutes post-intoxication and continued to increase throughout the first hour of intoxication (Fig. 6D). Similar results were also observed for phagocytes isolated from the peritoneum of mice after thioglycollate treatment, from the bone marrow of naïve mice, and from whole blood-derived primary human neutrophils (Data not shown and Fig. S2). Together, these results demonstrate that LukED is toxic to murine neutrophils due to membrane damage that leads to rapid cell death.

LukED targets and kills phagocytes in vivo

Although staphylococcal bi-component leukotoxins are known for their ability to kill immune cells *in vitro*, the mechanism by which these toxins contribute to *S. aureus* pathogenesis *in vivo* is poorly defined. We embarked on experiments to examine whether LukED promotes pathogenesis *in vivo* by killing phagocytes recruited to hematogenously-seeded infection sites (in this case, murine kidneys). Mice were infected with Newman wild type, Δ*lukED*, or Δ*lukED*::*lukED* strains and after 96 hours, kidneys were removed, and single cell suspensions prepared for flow cytometric analysis. Cells were stained with a fixable viability dye (PacBlue), and α-CD11b antibody (to detect phagocytes). ~40% of total cells (including kidney parenchymal cells as well as infiltrating immune cells) stained PacBlue$^+$ regardless of whether they were infected with WT, Δ*lukED*, or Δ*lukED*::*lukED* indicating similar sample processing for all organs (Fig. S3). Further analyses of CD11b$^+$/PacBlue$^+$ cells (total non-viable phagocytes) revealed a significant reduction in overall cell viability (~90% PacBlue$^+$) for both wild type and Δ*lukED*::*lukED* infected animals (Fig. 7A). In stark contrast, mice infected with the Δ*lukED* mutant exhibited greater proportions of viable phagocytes in infected kidneys (only ~50% PacBlue$^+$) (Fig. 7A). These results suggest that LukED directly impacts the viability of phagocytic cells at the site of tissue infection.

LukED promotes S. aureus *virulence* in vivo *by killing phagocytes*

If the primary contribution of LukED to *S. aureus* pathogenesis is neutrophil killing, depletion of neutrophils prior to infection should result in comparable virulence characteristics between wild type and the Δ*lukED* mutant strain. To test this hypothesis, we specifically depleted neutrophils using the 1A8 anti-Ly6G antibody (Daley *et al.*, 2008). The 1A8 antibody was efficient at depleting neutrophils (Gr-1$^+$/CD11b$^+$), while the 2A3 isotype control antibody was not (Fig. 7B) (Daley et al., 2008, Blomgran & Ernst, 2011). In contrast, the antibodies have no effect on lymphocytes (B cells CD3$^-$/B220$^+$; T cells B220$^-$/CD3$^+$) (Fig. 7B). Following antibody administration, mice were infected systemically with ~1×10^8 CFU wild type or the Δ*lukED* mutant. Animals treated with 2A3 (isotype control antibody) exhibited survival patterns similar to those already described, confirming that *lukED* is critical for the full virulence of *S. aureus*. In contrast, when mice were depleted of neutrophils, the virulence of the wild type and the Δ*lukED* mutant were indistinguishable (Fig. 7C). In this experiment, we observed that administration of 2A3 control antibody resulted in slower kinetics of animal death after infection with wild type compared to neutrophil-depleted animals, an effect presumably due to subtle influences of the control antibody on the murine immune response. It thus remained possible that an infectious dose of *S. aureus* resulting in 100% lethality within 30 hours might also lack a distinguishable phenotype between wild type and a Δ*lukED* mutant irrespective of the presence/absence of neutrophils. To rule out this possibility we also infected untreated animals with 1×10^8 CFU of *S. aureus* Newman, and Δ*lukED,* and measured survival over time (Fig. 7C). The majority of animals (5 out of 6) infected with wild type rapidly succumbed to infection within 36 hours, while those infected with a Δ*lukED* mutant remained markedly attenuated. Collectively, these results demonstrate that LukED targets neutrophils *in vivo* to promote *S. aureus* virulence.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Discussion

To cause severe disease *S. aureus* must efficiently avoid rapid killing by host neutrophils, which mediate the initial response to infection. The mechanism(s) by which the bacterium averts neutrophil killing is multi-faceted and incompletely understood, but is believed to rely heavily upon secreted proteins that can inhibit the function of and/or kill these critical immune cells (Wang *et al.*, 2007, Dumont et al., 2011). In this study, we conclusively demonstrate that LukED contributes to the pathophysiology of *S. aureus* by killing neutrophils *in vivo* facilitating bacterial growth at the site of infection. Thus, our findings extend the complex and integrated role of toxins in *S. aureus* immune cell killing and highlight LukED as a critical virulence factor involved in the lethality observed in *S. aureus* bacteremia.

Why LukED had not been previously implicated as a major virulence factor in *S. aureus* is not certain, though we speculate it may stem from the redundant cytotoxic activities of toxins present in *S. aureus* culture supernatant toward human phagocytes (Wang et al., 2007, Malachowa et al., 2011, Ventura et al., 2010, Dumont et al., 2011). In addition, the expression and production of leukotoxins in *S. aureus* is heavily influenced by growth medium and growth conditions, which in turn, modulate the cytotoxicity of *S. aureus* culture supernatants (Malachowa et al., 2011). Such findings suggest caution in the interpretation of *in vitro* studies using culture supernatants. An advantage of the study design implemented in this work is its minimal reliance on *ex vivo* and *in vitro* phenotypic analyses to infer *in vivo* functionality. LukED is thus far the only *Staphylococcal* leukotoxin found to exhibit potency toward murine phagocytes *ex vivo* and *in vivo*. Thus, the utility of LukED-based studies using mouse models will certainly prove an advantageous means by which to further elucidate the true functional role of bi-component leukotoxins during host infection.

Both LukE and LukD are 100% conserved at the amino acid level in sequenced *S. aureus* strains (Fig. S4), suggesting that the major biological function of the toxin is similar to that described in this study. Previous reports indicate the existence of a variant LukED toxin (LukEDv) (Morinaga et al., 2003). Upon closer examination we have confirmed that the "variant" sequence is conserved in nearly all sequenced strains, including *S. aureus* Newman (Fig S4). Contrary to the originally described *lukE/D* sequences in strain Newman (Gravet et al., 1998), the sequences of *lukE/D* in the Newman genome sequence (Accession #: NC_009641) are 100% identical to *lukED*v (Fig S4) (Baba *et al.*, 2008, Morinaga et al., 2003). We thus propose that LukED and LukEDv are in essence one and the same.

The *lukE/D* genes are present in ~87% of tested strains, including MSSA and MRSA (Gravet et al., 1998, Gravet *et al.*, 1999, Gravet *et al.*, 2001, Morinaga et al., 2003, Diep et al., 2006), underscoring its potential pivotal role in pathogenesis. Consistent with this observation, antibodies directed against LukED have been found in patients suffering from diverse *S. aureus* infections (Verkaik *et al.*, 2010), suggesting that LukED is produced during the course of human infection. Additionally, epidemiological evidence links *lukED* to *S. aureus* associated impetigo and diarrhea (Gravet *et al.*, 2001, Gravet *et al.*, 1999). It remains to be determined whether MRSA strains other than USA500 (e.g. USA100, USA300, and USA400) rely as heavily upon LukED for systemic infection. However, the observation that deletion of *lukED* significantly attenuates highly virulent strains of *S. aureus* supports the premise that selectively inhibiting LukED may prove valuable in the development of novel treatment strategies to combat *S. aureus* systemic infection.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Experimental Procedures

Bacterial strains and culture conditions

S. aureus strains used in this work are described in Table 1 and 2. Cultures were grown in either tryptic soy broth (TSB), or RPMI supplemented with 1% casamino acids as describe previously (Torres *et al.*, 2010, Dumont et al., 2011). Overnight cultures were routinely incubated at 37°C with shaking (180 RPM) and subcultured 1:100 for 3 to 5 hours under these same conditions. Due to the lack of antibiotic selection strategies for the USA500 strains we were unable to utilize available tools to complement Δ*lukED* mutants. Thus, construction of two independent mutants was used to validate the phenotype of USA500 (Fig. 3D).

Generation of mutant and complemented strains

All mutants not previously described were constructed via transduction of marked mutations using phage 80α (Table 2). Mutant strains (*lukED*∷kan) were generated using the allelic replacement strategy previously described (Bae & Schneewind, 2006). Plasmids for allelic replacement of *lukED* were constructed using pCR2.1 and pKOR-1. A kanamycin resistance cassette (*aphA3*) was amplified from plasmid pBT-K (kindly provided to us by Dr. Anthony Richardson) using oligonucleotide pair VJT524 (5′-TCCCCCCGGG-CTTTTTAGACATCTAAATCTAGGTAC) and VJT525 (5′-TCCCCCCGGG-CTCGACGATAAACCCAGCGAAC) and subsequently digested with XmaI and subcloned into the pCR2.1 vector containing sequences flanking the *lukED* locus (an internal XmaI site was previously generated between both flanking sequences to facilitate the insertion of antibiotic resistance markers). A PCR amplicon of the resultant *lukED* flanking sequences containing the internal kanamycin resistance gene was then recombined into pKOR1 resulting in the pKOR-1Δ*lukED*∷*kan* plasmid. Further allelic replacement was carried out in strain Newman according to previously described methods and subsequently introduced into all other strains via transduction.

A *lukED* complementation strain was generated by cloning into plasmid pJC1112, which stably integrates into the SaPI-1 site of *S. aureus* resulting in single copy chromosomal complementation. To construct pJC1112, plasmid pJC1001, which carries the SaPI 1 attachment site on a temperature sensitive pT181 (cop634) replicon was digested with HpaI and subsequently re-ligated thereby removing the pT181 replicon and making it a suicide plasmid in *S. aureus*. To construct the pJC1112-*lukED* complementation vector, a PCR amplicon containing the *lukED* operon and upstream 791 bp was generated using primer pairs VJT605 (5′-CCCC-CTGCAG(PstI)-GATAGGTGAGATGCATACACAAC) and VJT299 (5′-CCCC-GGATCC(BamHI)-TTA-TACTCCAGGATTAGTTTCTTTAG) and was subsequently digested and subcloned into pJC1112. The resultant plasmid was designated pJC1112-*lukED* and was subsequently integrated into the *S. aureus* SaPI-1 site.

Murine systemic infection with S. aureus

All animal infections were performed according to protocols approved by the NYU School of Medicine Institutional Animal Care and use Committee. Female ND4 Swiss Webster mice (~6-weeks old) (Harlan laboratories) were used in all experiments (Dumont et al., 2011). Mice were first anesthetized via intraperitoneal injection with 250μl Avertin (2,2,2-tribromoethanol dissolved in 2-methyl-2-butanol and diluted to a final concentration of 2.5% v/v in sterile saline) followed by infection via the retro-orbital venous plexus with 100μl PBS containing ~1×10^7 colony forming units (CFU) of *S. aureus* (Dumont et al., 2011) except for neutrophil depletion studies in which ~1×10^8 CFU were injected. For "survival" curves, mice were observed at 3-5 hour intervals and examined for signs of morbidity (hunched posture, ruffled fur, lack of movement, paralysis, and an inability to acquire food/

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

water). At these prescribed end points mice were immediately sacrificed and survival curves were plotted over time. To measure bacterial burden to infected kidneys, mice were sacrificed at 16 or 96 hours post infection and kidneys were isolated, homogenized and serial dilutions were plated onto tryptic soy agar (TSA) plates to enumerate CFU. For all other mouse experiments animals were sacrificed at either 16 or 96 hours post-infection and tissue/blood samples were collected for processing. All animal experiments were performed at least twice with groups of six or greater animals (see figure legends for specific cohort sizes).

Serum cytokines/chemokines

Serum was collected from mice infected as described above and IL-6 and GCSF were quantified using cytometric bead arrays (Becton Dickson; BD).

Isolation of PECS and intoxications

Mice were injected intraperitoneally with 1×10^7 CFU of *S. aureus* strain Newman. 16 hours post-injection, the peritoneal cavity was flushed with PBS containing gentamicin (50µg/ml), penicillin (100µg/ml), and streptomycin (100 µg/ml). Isolated PECs were subsequently washed, filtered, counted with trypan blue exclusion, and intoxicated with purified recombinant LukED followed by antibody staining for FACS analysis. Cell viability, membrane permeability, and pore formation were measured via CellTiter (Promega), CytotoxOne (Promega), and EtBr incorporation, respectively, on an EnVision 2103 plate reader (Perkin-Elmer). Light and fluorescent microscopy images were acquired using an Axiovert 40CFL microscope (Zeiss).

Characterization of primary immune cells

Kidneys from infected mice were dissected and single cell suspensions generated (Torres *et al.*, 2007). Cells were incubated with CD16/CD32 Fc blocker and subsequently stained with the following antibodies and dyes at the described dilutions: pacific blue viability dye (1:1000; Invitrogen), α-CD11b-PE-Cy7 (1:200; BD), and α-CD3-APC (1:250; BD). For characterization of PECs, cells were processed and blocked as described above and subsequently stained with the following antibodies: anti-CD11b-PE-Cy7 (1:200), and anti-Ly6G-FITC (1:500; BD). All samples were analyzed on an LSR-II flow cytometer (Bectin-Dickson, BD). For quantitation of immune cells isolated from infected kidneys a total of five independent mice were infected with each strain and percentages of cells acquired were averaged. Peritoneal elicited cells were isolated on two independent occasions from six mice and intoxications were subsequently conducted in triplicate at each toxin dose.

in vivo *neutrophil depletion studies*

Groups of six mice were injected with 300µg of either anti-Ly6G (1A8) antibody or an isotype control (2A3) antibody intraperitoneally 48 hours prior to infection as described previously (Blomgran & Ernst, 2011). 1×10^8 CFU of either wild type (Newman) or Δ*lukED* was injected retro-orbitally and "survival" was monitored over time. Spleens from control animals infected for 16 hours with *S. aureus* were isolated, stained with anti-GR1-PE (1:1500; BD), anti-CD11b-PE-Cy7 (1:200), anti-CD3-APC (1:250), and anti-B220-FITC (1:500; BD) antibodies, and analyzed by FACS to confirm neutrophil depletion in 1A8, but not 2A3 treated animals.

Generation of α- LukE and HlgC polyclonal sera

Rabbit polyclonal α-LukE and α-HlgC sera were generated using recombinant proteins as previously described (Dumont et al., 2011).

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Protein purification

6X-His-LukE and 6X-His-LukD single subunit expression vectors were kindly provided by Dr. Naoko Morinaga (Chiba University, Japan) and subsequently transformed into the *Escherichia coli* LysYLaqQ expression strain (New England Biolabs). 800 ml cultures were incubated at 37°C, 220RPM for 3.5 hours followed by cooling to 16°C and induction with 0.1mM IPTG for 16 hours at 16°C, 220 RPM. Bacterial pellets were sonicated on ice and cell lysates clarified by centrifugation at 10000 RPM for 30 minutes followed by incubation with 1ml of Ni-NTA resin for 1hour. Bound protein was washed and eluted with tris-buffered saline (TBS) supplemented with 500mM Imidazole and subsequently dialyzed into TBS + 10% glycerol. 100μl aliquots of filter sterilized protein were stored at −80°C until use.

Western blot

Strains were grown as described above. Cell-free culture supernatants containing soluble secreted proteins were subsequently collected, filter-sterilized, and the proteins precipitated with TCA as described previously (Dumont et al., 2011). All protein samples were run on 10% SDS-PAGE gels at 80V for approximately 3 hours. Proteins were transferred to nitrocellulose at 1 Amp for 1 hour followed by blocking in phosphate buffered saline containing 0.1% Tween (PBST). Primary antibody dilutions were as follows: LukA (1:5000), LukE (1:10000), HlgC (1:5000), and Hla (1:5000; Sigma). Mouse anti-rabbit secondary antibody conjugated to AlexaFluor-680 was used at a 1:25000 dilution. Western blots were scanned on an Odyssey Imager (Licor).

Quantitative reverse transcriptase PCR (qRT-PCR)

Total RNA was prepared from the indicated *S. aureus* strains (Table 1) grown for 5 hours in RPMI to an OD of ~1.3. 20 ml of bacterial culture was subsequently mixed 1:1 with a solution of 50% ethanol/50% acetone and frozen at −80°C until use. RNA was extracted from bacterial cells using an RNeasy purification kit according to the manufacturers protocol (Qiagen). Quality of the RNA was evaluated on an agarose-formaldehyde gel, and RNA abundance quantified using a nanodrop spectrophotometer. A SYBER green-based (Qiagen) ddCt relative quantitation of gene expression assay was set up using 16S rRNA amplification as the endogenous control for all samples as described previously (Benson *et al.*, 2011). The following primers were used: 16s rRNA (TGAGATGTTGGGTTAAGTCCCGCA, CGGTTTCGCTGCCCTTTGTATTGT) and *lukE* (GAAATGGGGCGTTACTCAAA, GAATGGCCAAATCATTCGTT). Dissociation curves were determined for each primer set and relative quantitation of gene expression was determined for duplicate reactions of each strain tested using Applied Biosystems Real Time Quantitative PCR Software (Applied Biosystems). Relative gene expression was determined by comparing all strains to the isogenic Newman *lukED* knockout strain that produces no transcript.

Statistics

Statistics for survival curves was calculated using a Log-Rank (Mantel-Cox) test for statistical significance between curves. For all other experiments either 1-way ANOVA with Tukey's multiple comparison test, or a two-tailed Student's *t*-test was used to determine statistical significance between groups. In all cases a p value of less than 0.05 was considered statistically meaningful.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Acknowledgments

We thank members of the Torres Laboratory, Dr. Nancy Freitag, and Dr. Joel Ernst for critically reading this manuscript. We are grateful to Dr. Naoko Morinaga for the kind gift of the LukE and LukD expression vectors; Dr. Timothy Foster for the gift of the Δ*hla* and Δ*hlg* mutant *S. aureus* strains, Drs. Joel Ernst and Ludovic Desvignes for help with the neutrophil depletion studies, and Lina Kozhaya and Stephen Rawlings for assistance in setting up FACS experiments. Several of the *S. aureus* strains used in this work were obtained from the NIH-NIAID supported Network on Antimicrobial Resistance in *S. aureus* (NARSA). This research was supported in part by New York University School of Medicine Development Funds (VJT), grant 1R56AI091856-01A1 from the National Institute of Allergy and Infectious Diseases (VJT), grant 5R01AI022159-26 from the National Institute of Allergy and Infectious Diseases (RPN), and an American Heart Association Scientist Development Grant (09SDG2060036) (VJT). MAB was supported in part by an American Heart Association predoctoral fellowship (10PRE3420022). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

Methicillin-resistant Staphylococcus aureus infections in correctional facilities---Georgia. Vol. 52. MMWR Morb Mortal Wkly Rep; California, and Texas: 2003. p. 992-996.2001-2003

Baba T, Bae T, Schneewind O, Takeuchi F, Hiramatsu K. Genome sequence of Staphylococcus aureus strain Newman and comparative analysis of staphylococcal genomes: polymorphism and evolution of two major pathogenicity islands. J Bacteriol. 2008; 190:300–310. [PubMed: 17951380]

Bae T, Schneewind O. Allelic replacement in Staphylococcus aureus with inducible counter-selection. Plasmid. 2006; 55:58–63. [PubMed: 16051359]

Benson MA, Lilo S, Wasserman GA, Thoendel M, Smith A, Horswill AR, Fraser J, Novick RP, Shopsin B, Torres VJ. Staphylococcus aureus regulates the expression and production of the staphylococcal superantigen-like secreted proteins in a Rot-dependent manner. Mol Microbiol. 2011

Blomgran R, Ernst JD. Lung neutrophils facilitate activation of naive antigen-specific CD4+ T cells during Mycobacterium tuberculosis infection. J Immunol. 2011; 186:7110–7119. [PubMed: 21555529]

Bodey GP, Buckley M, Sathe YS, Freireich EJ. Quantitative relationships between circulating leukocytes and infection in patients with acute leukemia. Ann Intern Med. 1966; 64:328–340. [PubMed: 5216294]

Boisset S, Geissmann T, Huntzinger E, Fechter P, Bendridi N, Possedko M, Chevalier C, Helfer AC, Benito Y, Jacquier A, Gaspin C, Vandenesch F, Romby P. Staphylococcus aureus RNAIII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism. Genes Dev. 2007; 21:1353–1366. [PubMed: 17545468]

Dale DC, Guerry D. t. Wewerka JR, Bull JM, Chusid MJ. Chronic neutropenia. Medicine (Baltimore). 1979; 58:128–144. [PubMed: 431399]

Daley JM, Thomay AA, Connolly MD, Reichner JS, Albina JE. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. J Leukoc Biol. 2008; 83:64–70. [PubMed: 17884993]

Diep BA, Carleton HA, Chang RF, Sensabaugh GF, Perdreau-Remington F. Roles of 34 virulence genes in the evolution of hospital- and community-associated strains of methicillin-resistant Staphylococcus aureus. J Infect Dis. 2006; 193:1495–1503. [PubMed: 16652276]

Diep BA, Chan L, Tattevin P, Kajikawa O, Martin TR, Basuino L, Mai TT, Marbach H, Braughton KR, Whitney AR, Gardner DJ, Fan X, Tseng CW, Liu GY, Badiou C, Etienne J, Lina G, Matthay MA, DeLeo FR, Chambers HF. Polymorphonuclear leukocytes mediate Staphylococcus aureus Panton-Valentine leukocidin-induced lung inflammation and injury. Proc Natl Acad Sci U S A. 2010; 107:5587–5592. [PubMed: 20231457]

Dumont AL, Nygaard TK, Watkins RL, Smith A, Kozhaya L, Kreiswirth BN, Shopsin B, Unutmaz D, Voyich JM, Torres VJ. Characterization of a new cytotoxin that contributes to Staphylococcus aureus pathogenesis. Mol Microbiol. 2011; 79:814–825. [PubMed: 21255120]

Duthie ES, Lorenz LL. Staphylococcal coagulase; mode of action and antigenicity. J Gen Microbiol. 1952; 6:95–107. [PubMed: 14927856]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Finck-Barbancon V, Duportail G, Meunier O, Colin DA. Pore formation by a two-component leukocidin from Staphylococcus aureus within the membrane of human polymorphonuclear leukocytes. Biochim Biophys Acta. 1993; 1182:275–282. [PubMed: 8399361]

Foster TJ. Immune evasion by staphylococci. Nat Rev Microbiol. 2005; 3:948–958. [PubMed: 16322743]

Geisinger E, Adhikari RP, Jin R, Ross HF, Novick RP. Inhibition of rot translation by RNAIII, a key feature of agr function. Mol Microbiol. 2006; 61:1038–1048. [PubMed: 16879652]

Gravet A, Colin DA, Keller D, Girardot R, Monteil H, Prevost G. Characterization of a novel structural member, LukE-LukD, of the bi-component staphylococcal leucotoxins family. FEBS Lett. 1998; 436:202–208. [PubMed: 9781679]

Gravet A, Couppie P, Meunier O, Clyti E, Moreau B, Pradinaud R, Monteil H, Prevost G. Staphylococcus aureus isolated in cases of impetigo produces both epidermolysin A or B and LukE-LukD in 78% of 131 retrospective and prospective cases. J Clin Microbiol. 2001; 39:4349–4356. [PubMed: 11724844]

Gravet A, Rondeau M, Harf-Monteil C, Grunenberger F, Monteil H, Scheftel JM, Prevost G. Predominant Staphylococcus aureus isolated from antibiotic-associated diarrhea is clinically relevant and produces enterotoxin A and the bicomponent toxin LukE-lukD. J Clin Microbiol. 1999; 37:4012–4019. [PubMed: 10565923]

Kennedy AD, Otto M, Braughton KR, Whitney AR, Chen L, Mathema B, Mediavilla JR, Byrne KA, Parkins LD, Tenover FC, Kreiswirth BN, Musser JM, DeLeo FR. Epidemic community-associated methicillin-resistant Staphylococcus aureus: recent clonal expansion and diversification. Proc Natl Acad Sci U S A. 2008; 105:1327–1332. [PubMed: 18216255]

Klevens RM, Edwards JR, Tenover FC, McDonald LC, Horan T, Gaynes R. Changes in the epidemiology of methicillin-resistant Staphylococcus aureus in intensive care units in US hospitals, 1992-2003. Clin Infect Dis. 2006; 42:389–391. [PubMed: 16392087]

Klevens RM, Morrison MA, Nadle J, Petit S, Gershman K, Ray S, Harrison LH, Lynfield R, Dumyati G, Townes JM, Craig AS, Zell ER, Fosheim GE, McDougal LK, Carey RB, Fridkin SK. Invasive methicillin-resistant Staphylococcus aureus infections in the United States. Jama. 2007; 298:1763–1771. [PubMed: 17940231]

Labandeira-Rey M, Couzon F, Boisset S, Brown EL, Bes M, Benito Y, Barbu EM, Vazquez V, Hook M, Etienne J, Vandenesch F, Bowden MG. Staphylococcus aureus Panton-Valentine leukocidin causes necrotizing pneumonia. Science. 2007; 315:1130–1133. [PubMed: 17234914]

Lekstrom-Himes JA, Gallin JI. Immunodeficiency diseases caused by defects in phagocytes. The New England journal of medicine. 2000; 343:1703–1714. [PubMed: 11106721]

Li M, Cheung GY, Hu J, Wang D, Joo HS, Deleo FR, Otto M. Comparative analysis of virulence and toxin expression of global community-associated methicillin-resistant Staphylococcus aureus strains. J Infect Dis. 2010; 202:1866–1876. [PubMed: 21050125]

Li M, Diep BA, Villaruz AE, Braughton KR, Jiang X, DeLeo FR, Chambers HF, Lu Y, Otto M. Evolution of virulence in epidemic community-associated methicillin-resistant Staphylococcus aureus. Proc Natl Acad Sci U S A. 2009; 106:5883–5888. [PubMed: 19293374]

Loffler B, Hussain M, Grundmeier M, Bruck M, Holzinger D, Varga G, Roth J, Kahl BC, Proctor RA, Peters G. Staphylococcus aureus panton-valentine leukocidin is a very potent cytotoxic factor for human neutrophils. PLoS Pathog. 2010; 6:e1000715. [PubMed: 20072612]

Malachowa N, Whitney AR, Kobayashi SD, Sturdevant DE, Kennedy AD, Braughton KR, Shabb DW, Diep BA, Chambers HF, Otto M, Deleo FR. Global Changes in Staphylococcus aureus Gene Expression in Human Blood. PLoS One. 2011; 6:e18617. [PubMed: 21525981]

McNamara PJ, Bayer AS. A rot mutation restores parental virulence to an agr-null Staphylococcus aureus strain in a rabbit model of endocarditis. Infect Immun. 2005; 73:3806–3809. [PubMed: 15908418]

McNamara PJ, Milligan-Monroe KC, Khalili S, Proctor RA. Identification, Cloning, and Initial Characterization of rot, a Locus Encoding a Regulator of Virulence Factor Expression in *Staphylococcus aureus*. J Bacteriol. 2000; 182:3197–3203. [PubMed: 10809700]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Menestrina G, Dalla Serra M, Comai M, Coraiola M, Viero G, Werner S, Colin DA, Monteil H, Prevost G. Ion channels and bacterial infection: the case of beta-barrel pore-forming protein toxins of Staphylococcus aureus. FEBS Lett. 2003; 552:54–60. [PubMed: 12972152]

Morinaga N, Kaihou Y, Noda M. Purification, cloning and characterization of variant LukE-LukD with strong leukocidal activity of staphylococcal bi-component leukotoxin family. Microbiol Immunol. 2003; 47:81–90. [PubMed: 12636257]

Nilsson IM, Hartford O, Foster T, Tarkowski A. Alpha-toxin and gamma-toxin jointly promote Staphylococcus aureus virulence in murine septic arthritis. Infect Immun. 1999; 67:1045–1049. [PubMed: 10024541]

Nizet V. Understanding how leading bacterial pathogens subvert innate immunity to reveal novel therapeutic targets. J Allergy Clin Immunol. 2007; 120:13–22. [PubMed: 17606031]

Novick RP, Geisinger E. Quorum sensing in staphylococci. Annu Rev Genet. 2008; 42:541–564. [PubMed: 18713030]

Novick RP, Ross HF, Projan SJ, Kornblum J, Kreiswirth B, Moghazeh S. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. Embo J. 1993; 12:3967–3975. [PubMed: 7691599]

Pincus SH, Boxer LA, Stossel TP. Chronic neutropenia in childhood. Analysis of 16 cases and a review of the literature. Am J Med. 1976; 61:849–861. [PubMed: 795298]

Roberts RB, de Lencastre A, Eisner W, Severina EP, Shopsin B, Kreiswirth BN, Tomasz A, MRSA Collaborative Study Group. Molecular epidemiology of methicillin-resistant Staphylococcus aureus in 12 New York hospitals. J Infect Dis. 1998; 178:164–171. [PubMed: 9652436]

Said-Salim B, Dunman PM, McAleese FM, Macapagal D, Murphy E, McNamara PJ, Arvidson S, Foster TJ, Projan SJ, Kreiswirth BN. Global Regulation of *Staphylococcus aureus* Genes by Rot. J Bacteriol. 2003; 185:610–619. [PubMed: 12511508]

Supersac G, Piemont Y, Kubina M, Prevost G, Foster TJ. Assessment of the role of gamma-toxin in experimental endophthalmitis using a hlg-deficient mutant of Staphylococcus aureus. Microb Pathog. 1998; 24:241–251. [PubMed: 9533895]

Torres VJ, Attia AS, Mason WJ, Hood MI, Corbin BD, Beasley FC, Anderson KL, Stauff DL, McDonald WH, Zimmerman LJ, Friedman DB, Heinrichs DE, Dunman PM, Skaar EP. Staphylococcus aureus fur regulates the expression of virulence factors that contribute to the pathogenesis of pneumonia. Infect Immun. 2010; 78:1618–1628. [PubMed: 20100857]

Torres VJ, Stauff DL, Pishchany G, Bezbradica JS, Gordy LE, Iturregui J, Anderson KL, Dunman PM, Joyce S, Skaar EP. A Staphylococcus aureus regulatory system that responds to host heme and modulates virulence. Cell Host Microbe. 2007; 1:109–119. [PubMed: 18005689]

Ventura CL, Malachowa N, Hammer CH, Nardone GA, Robinson MA, Kobayashi SD, DeLeo FR. Identification of a novel Staphylococcus aureus two-component leukotoxin using cell surface proteomics. PLoS One. 2010; 5:e11634. [PubMed: 20661294]

Verkaik NJ, Dauwalder O, Antri K, Boubekri I, de Vogel CP, Badiou C, Bes M, Vandenesch F, Tazir M, Hooijkaas H, Verbrugh HA, van Belkum A, Etienne J, Lina G, Ramdani-Bouguessa N, van Wamel WJ. Immunogenicity of toxins during Staphylococcus aureus infection. Clin Infect Dis. 2010; 50:61–68. [PubMed: 19947854]

Voyich JM, Braughton KR, Sturdevant DE, Whitney AR, Said-Salim B, Porcella SF, Long RD, Dorward DW, Gardner DJ, Kreiswirth BN, Musser JM, Deleo FR. Insights into Mechanisms Used by Staphylococcus aureus to Avoid Destruction by Human Neutrophils. J Immunol. 2005; 175:3907–3919. [PubMed: 16148137]

Voyich JM, Otto M, Mathema B, Braughton KR, Whitney AR, Welty D, Long RD, Dorward DW, Gardner DJ, Lina G, Kreiswirth BN, DeLeo FR. Is Panton-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant Staphylococcus aureus disease? J Infect Dis. 2006; 194:1761–1770. [PubMed: 17109350]

Wang R, Braughton KR, Kretschmer D, Bach TH, Queck SY, Li M, Kennedy AD, Dorward DW, Klebanoff SJ, Peschel A, DeLeo FR, Otto M. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat Med. 2007; 13:1510–1514. [PubMed: 17994102]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

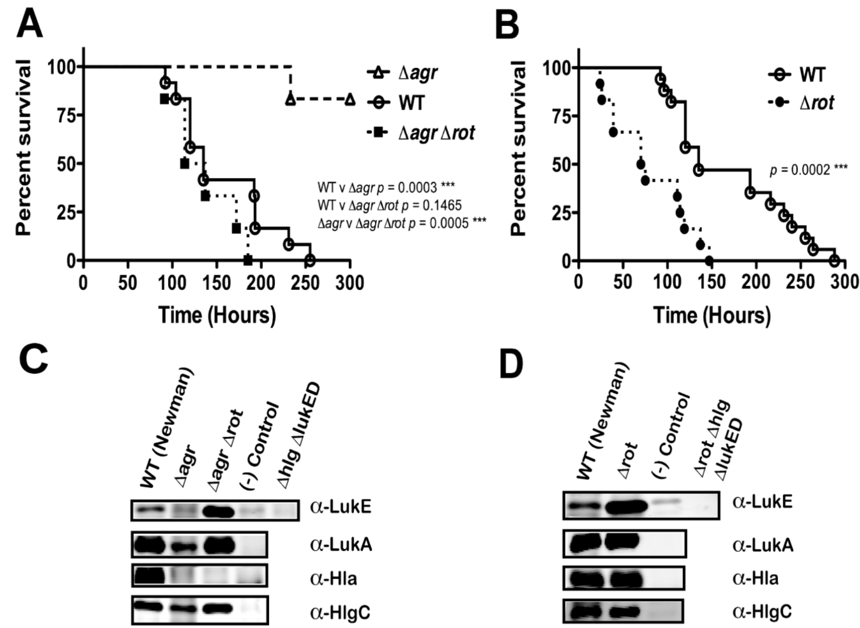

Figure 1. The regulatory input of Agr upon Rot directly influences bloodstream infection with *S. aureus*

(A-B) "Survival" of mice infected systemically with Newman wild type (WT), N=12 (A) and N=17 (B); Δ*agr*, N=6; Δ*agr* Δ*rot,* N=6; or Δ*rot*, N=12. Mice were injected with ~1×10^7 CFU of the indicated strains via the retro-orbital venous plexus and monitored for "survival" over time as described in *Experimental procedures.* (C-D) Immunoblots of leukotoxins produced by the indicated strains. (−) Control indicates proteins isolated from gene deletion mutants of the particular leukotoxin probed in each panel. For LukE immunoblots an Δ*hlg*Δ*lukED* or an Δ*rot*Δ*hlg*Δ*lukED* mutant was included due to cross reactivity of the anti-LukE antibody with subunits of γ-hemolysin. Statistically significant differences between curves were determined by Log-Rank (Mantel Cox) test and *p* values are shown (***, p≤0.0005).

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

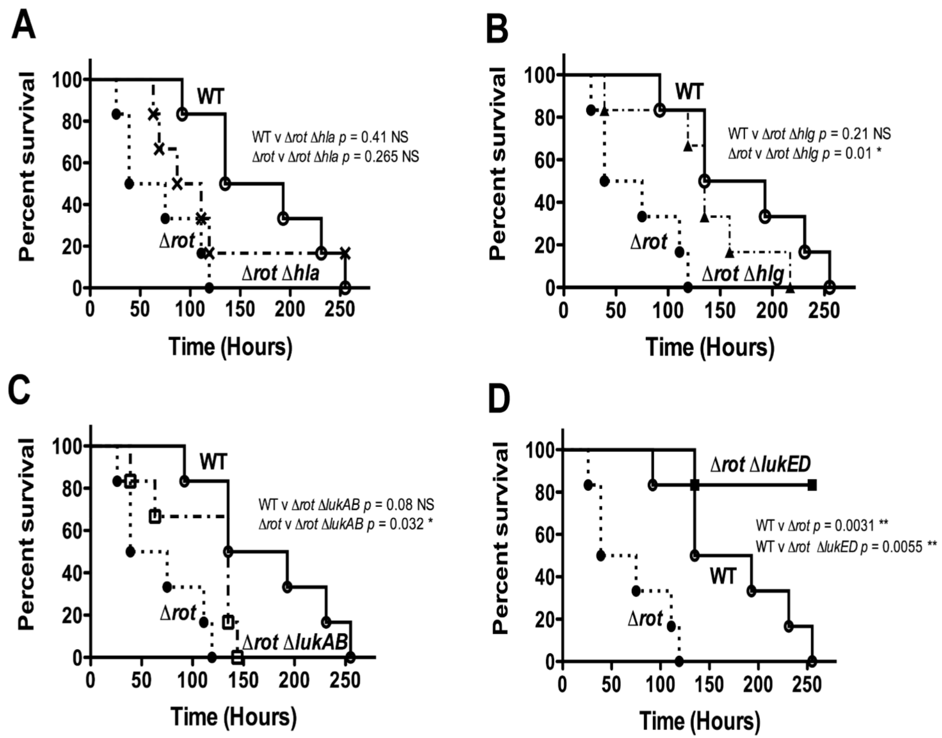

Figure 2. Identification of virulence factors critical to *S. aureus* bacteremia-mediated death

(A-D) "Survival" of mice infected with Newman wild type (WT), Δ*rot*, and *rot*/*leukotoxin* double mutants, all N=6. Mice were injected with ~1×10^7 CFU of the indicated strains via the retro-orbital venous plexus and monitored for "survival" over time as described in *Experimental procedures.* Statistically significant differences between curves were determined by Log-Rank (Mantel Cox) test and *p* values are shown (*, p<0.05; **, p≤0.005).

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

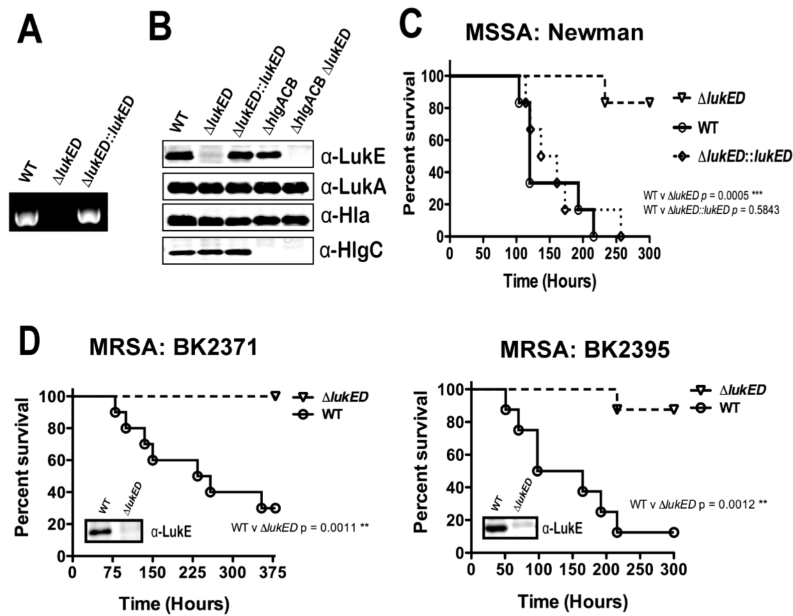

Figure 3. LukED contributes to lethality in *S. aureus* bloodstream infection

(A-B) PCR and immunoblot confirmation of the Newman Δ*lukED* mutant and its complement Δ*lukED*::*lukED*. For LukE immunoblots, an Δ*hlg*Δ*lukED* mutant was included due to cross reactivity of the anti-LukE antibody with subunits of γ-hemolysin. (C-D) "Survival" curves of MSSA (strain Newman) and MRSA (USA500 strains) infected mice. Mice were injected with ~1×10^7 CFU of Newman wild type (WT), N=6; Δ*lukED*, N=6; Δ*lukED*::*lukED*, N=6; USA500 (BK2371 or BK2395) wild type (WT), N=10; or an isogenic Δ*lukED* mutant from each USA500 strain, N=10, via the retro-orbital venous plexus and monitored for "survival" over time as described in *Experimental procedures.* Statistically significant differences between "survival" curves were determined by Log-Rank (Mantel Cox) test and *p* values are shown (**, p≤0.005; ***, p≤0.0005).

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

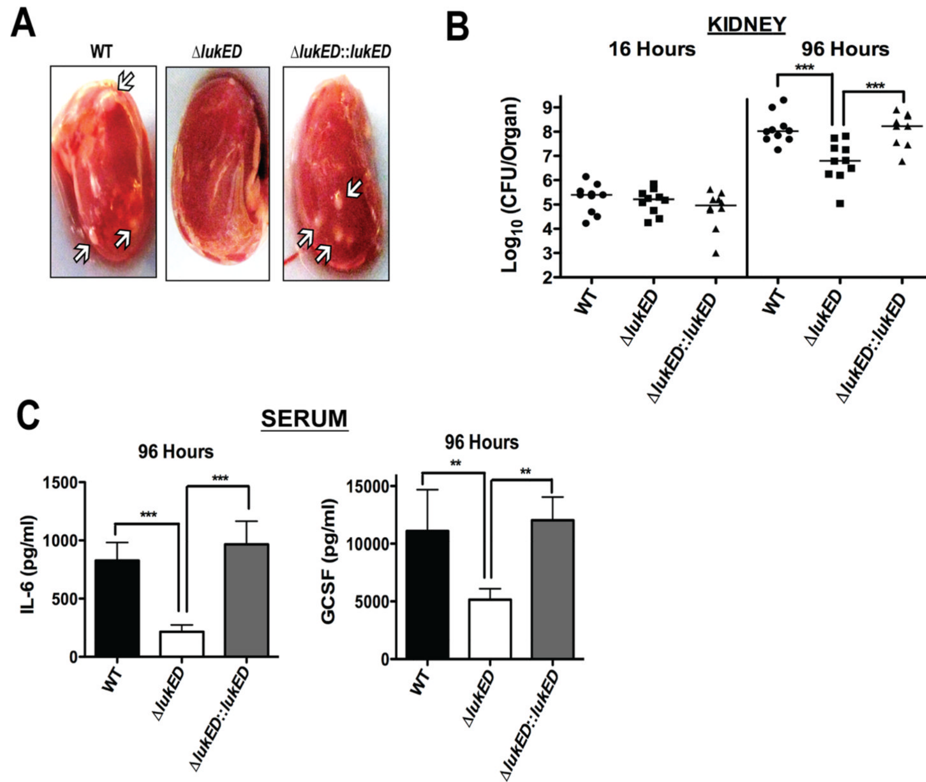

Figure 4. LukED facilitates bacterial replication *in vivo*

(A) Gross pathology of abscess formation in kidneys of mice infected for 96 hours with Newman wild type (WT), Δ*lukED*, and Δ*lukED*::*lukED*. White arrows point to abscesses in wild type and Δ*lukED*::*lukED* infected organs. (B) Enumeration of CFU from infected kidneys. Ten mice per group were injected with ~1×10^7 CFU of Newman wild type (WT), Δ*lukED*, and Δ*lukED*::*lukED*. After 96 hours, kidneys were removed and homogenized, followed by plating of serial dilutions onto solid media for enumeration of bacterial burden. (C) IL-6 and GCSF levels in the serum of animals from Panel B. Statistical significance between CFU and Serum cytokine levels was determined by 1-way-ANOVA with Tukey's multiple comparison test (**, p≤0.005; ***, p≤0.0005).

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

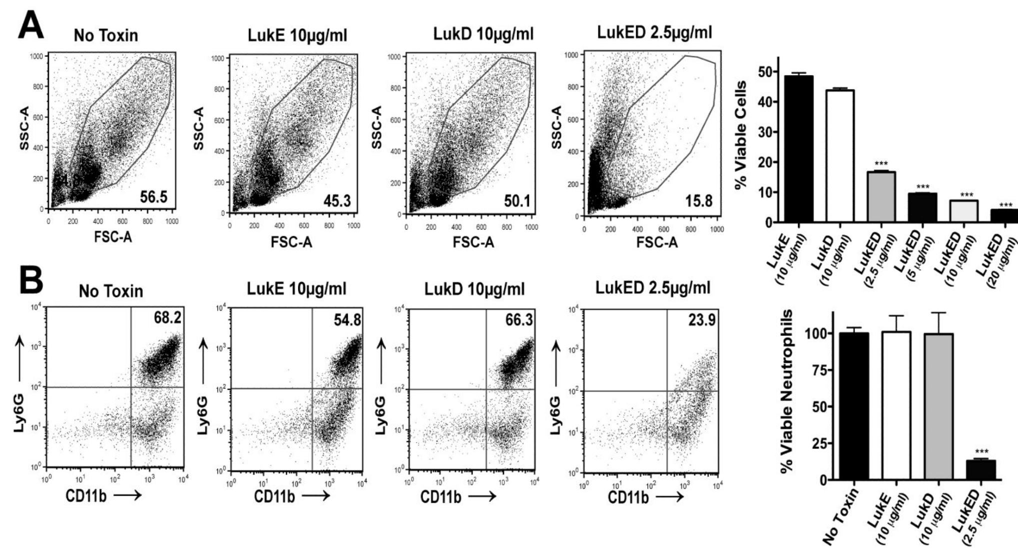

Figure 5. LukED kills phagocytes elicited to the site of infection *ex vivo*

(A) Profile of innate immune cells recruited to the peritoneum upon infection with *S. aureus* and subsequently intoxicated with the indicated amounts of LukE, LukD, or LukED. Representative FACS plots of cells in the live gate are shown. The graphical depiction represents the average percent viable PECs within the live gate. (B) Killing of primary murine neutrophils ($CD11b^+/Ly6G^+$) by LukED. Representative FACs plots are shown. The graphical depiction displays percent viable neutrophils relative to the total viable neutrophils recovered from unintoxicated controls (set to 100%). Statistical significance was determined by 1-way ANOVA with Tukey's multiple comparison test (***, $p \leq 0.0005$).

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

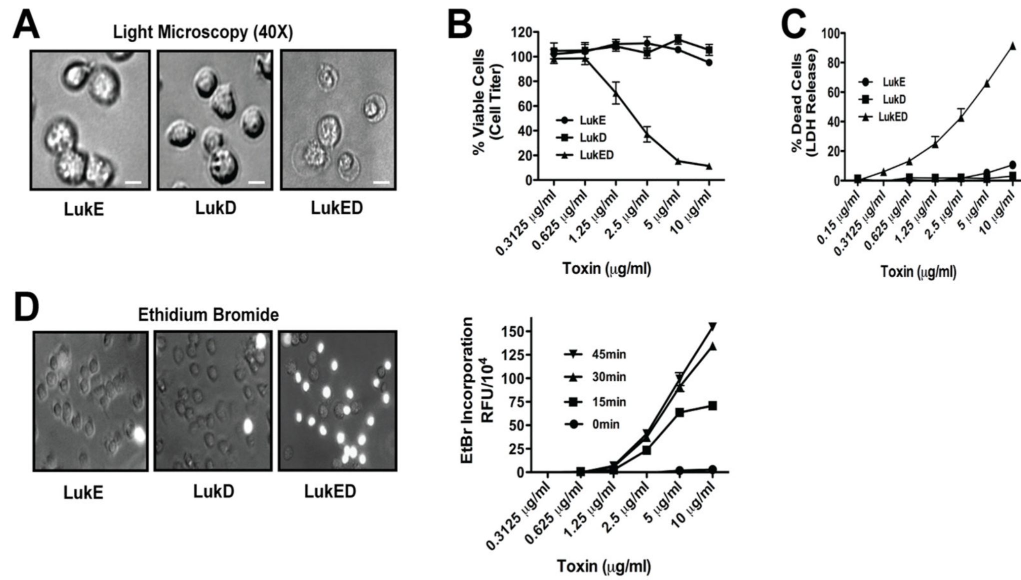

Figure 6. LukED targets and kills neutrophils by damaging their plasma membrane

Intoxicated PECs were evaluated for viability, membrane damage and pore formation via (A) light microscopy at 40X magnification, (B) metabolic activity (CellTiter), (C) LDH release (CytotoxOne), and (D) ethidium bromide incorporation into cellular DNA. For microscopic imaging of intoxicated cells (A and D) a toxin dose of 5 μg/ml was used. For all other assays of viability, membrane integrity, and pore formation a dose response of equimolar ratios of LukE and LukD was added to cells and measurements made as described in the *Experimental procedures*.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

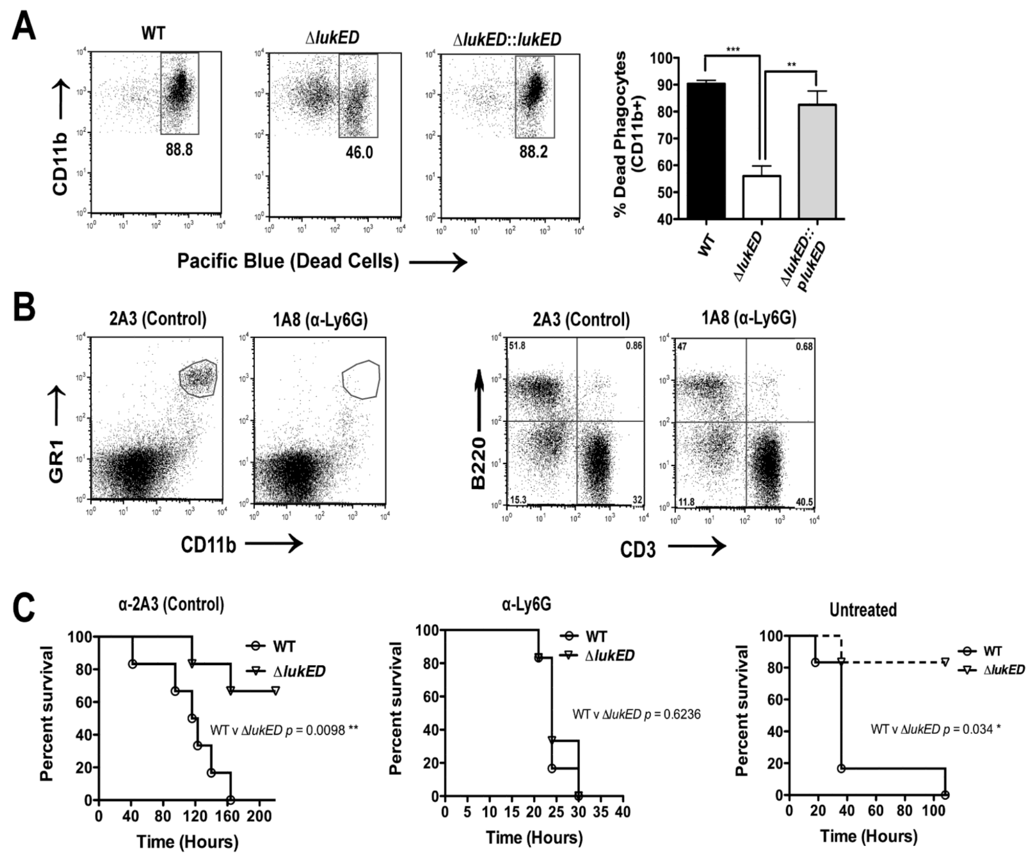

Figure 7. LukED promotes *S. aureus* virulence *in vivo* via its potent toxicity toward neutrophils
(A) Determination of percent viable CD11b+ phagocytic leukocytes in *S. aureus* infected kidneys. (B) Immune cell profile (neutrophils; GR1+/CD11b+, B lymphocytes CD3−/B220+, and T lymphocytes CD3+/B220−) of mice treated with a neutrophil depleting antibody (1A8) or an isotype control antibody (2A3) followed by systemic infection with wild type *S. aureus*. (C) "Survival" curves of mice treated with 2A3, 1A8, or untreated followed by systemic infection of ~1×10^8 CFU wild type (WT), N=10 for 2A3 or 1A8 and N=6 for untreated; or a Δ*lukED* mutant, N=10 for 2A3 or 1A8 and N=6 for untreated. Statistically significant differences between "survival" curves were determined by Log-Rank (Mantel Cox) test and *p* values are shown (*, $p<0.05$; **, $p\leq0.005$).

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Table 1

***S. aureus* clinical isolates used in this study**

Strain	Year	Specimen Source	PFGE type	*spa*-type	*spa* Motif	CC[a]	*agr* group	*lukED*	Citation
BK2382	1996	Wound	USA100	2	T1-J1-M1-B1-M1-D1-M1-G1-M1-K1	5	2	+	(Roberts *et al.*, 1998)
BK2405	1996	Tracheal aspirate	USA100	2	T1-J1-M1-B1-M1-D1-M1-G1-M1-K1	5	2	+	(Roberts *et al.*, 1998)
BK2516	1996	Wound	USA200	16	W1-G1-K1-A1-K1-A1-O1-M1-Q1-Q1-Q1	30	3	−	(Roberts *et al.*, 1998)
BK2532	1996	Sputum	USA200	16	W1-G1-K1-A1-K1-A1-O1-M1-Q1-Q1-Q1	30	3	−	(Klevens *et al.*, 2007)
LAC[b]	2002	Wound	USA300	1	Y1-H1-G1-F1-M1-B1-Q1-B1-L1-O1	8	1	+	(Kennedy *et al.*, 2008)
NRS647	2005	Blood	USA300	1	Y1-H1-G1-F1-M1-B1-Q1-B1-L1-O1	8	1	+	(Klevens *et al.*, 2007)
BK18810	2005	Pneumonia	USA300	1	Y1-H1-G1-F1-M1-B1-Q1-B1-L1-O1	8	1	+	(Kennedy *et al.*, 2008)
NRS193	1999	Pleural fluid	USA400	194	U1-J1-F1-K1-K1-P1-F1-K1-P1-E1	1	3	+	(2003)
MW2[c]	1998	Blood	USA400	131	U1-J1-J1-J1-J1-F1-E1	1	3	+	(2003)
BK2371	1996	Wound	USA500	7	Y1-H1-G1-C1-M1-B1-Q1-B1-L1-O1	8	1	+	(Roberts *et al.*, 1998)
BK2395	1996	Wound	USA500	7	Y1-H1-G1-C1-M1-B1-Q1-B1-L1-O1	8	1	+	(Roberts *et al.*, 1998)
Newman	1952	Osteomyelitis	N/A	1	Y1-H1-G1-F1-M1-B1-Q1-B1-L1-O1	8	1	+	(Duthie & Lorenz, 1952)

NOTE. −, absent; +, present; LAC, Los Angeles County clone; PFGE, pulse-field gel electrophoresis

[a] *spa*-type deduced clonal complex (CC). Isolates broadly grouped according to their relatedness by PFGE.

[b] Other designations: NRS384, NCBI reference sequence NC_007793

[c] Other designation: NRS123

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Table 2

***S. aureus* strains used in this study**

Strain	Background	Description	Designation	Reference
VJT21.44	*E. coli* LysY LaqQ	*Escherichia coli* LysY LaqQ + pET14b-6X-his-LukD		This Study
VJT20.78	*E. coli* LysYLaqQ	*Escherichia coli* LysY LaqQ + pET14b-6X-his-LukE		This Study
VJT3.81	*S. aureus* Newman	Parental Strain	WT (Newman)	(Duthie and Lorenz, 1952)
VJT7.17	*S. aureus* Newman	*S. aureus* Newman Δ*agr:tet*	Δ *agr*	(Benson et al., 2011)
VJT9.98	*S. aureus* Newman	*S. aureus* Newman Δ*rot::Tn917*	Δ *rot*	(Benson et al., 2011)
VJT10.03	*S. aureus* Newman	*S. aureus* Newman Δ*agr::tet* Δ*rot*::*Tn917*	Δ*agr* Δ*rot*	(Benson et al., 2011)
VJT8.16	*S. aureus* Newman	*S. aureus* Newman Δ*lukED*	Δ *lukED*	(Dumont et al., 2011)
VJT8.91	*S. aureus* Newman	*S. aureus* Newman Δ*lukAB*	Δ *lukAB*	(Dumont et al., 2011)
VJT7.12	*S. aureus* 8325-4	*S. aureus* 8325-4 *hla*::*erm hlb*::ϕ42E *hlg*::*tet*	Δ *hla* Δ *hlb* Δ *hlg*	(Nilsson et al., 1999)
VJT7.09	*S. aureus* Newman	*S. aureus* Newman Δ*hlgACB*::*tet*	Δ *hlgACB*	(Supersac et al., 1998)
VJT23.61	*S. aureus* Newman	*S. aureus* Newman Δ*lukED*::pJC1.112l*ukED*	Δ *lukED* :: *plukED*	This Study
VJT15.93	*S. aureus* Newman	*S. aureus* Newman Δ*rot*::*Tn917* Δ*lukAB*	Δrot AlukAB	This Study
VJT18.96	*S. aureus* Newman	*S. aureus* Newman Δ*rot*::*spec* Δ*hlg*::*tet*	Δ*rot* Δ*hlg*	This Study
VJT21.37	*S. aureus* Newman	*S. aureus* Newman Δ*rot*::*spec* Δ*hla:erm*	Δ*rot* Δ*hla*	This Study
VJT15.96	*S. aureus* Newman	*S. aureus* Newman Δ*rot*::*Tn917* Δ*lukED*	Δ*rot* Δ*lukED*	This Study
VJT21.92	*S. aureus* Newman	*S. aureus* Newman Δ*lukED* Δ*hlg*::*tet*	Δ*lukED* Δ*hlg*	This Study
VJT21.93	*S. aureus* Newman	*S. aureus* Newman Δ*rot*::*erm* Δ*lukED* Δ*hlg*::*tet*	Δ*rot* Δ*lukED* Δ*hlg*	This Study
VJT27.33	*S. aureus* USA500	USA500 BK2371	WT (USA500-1)	(Roberts et al., 1998)
VJT27.40	*S. aureus* USA500	USA500 BK2371 Δ*lukED*::*kan*	BK2371 Δ*lukED*	This Study
VJT27.36	*S. aureus* USA500	USA500 BK2395	WT (USA500-2)	(Roberts et al., 1998)
VJT27.43	*S. aureus* USA500	USA500 BK2395 Δ*lukED*::*kan*	BK2395 Δ*lukED*	This Study