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# RpiRc Is a Pleiotropic Effector of Virulence Determinant Synthesis and Attenuates Pathogenicity in *Staphylococcus aureus*

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**In *Staphylococcus aureus*, metabolism is intimately linked with virulence determinant biosynthesis, and several metabolite-responsive regulators have been reported to mediate this linkage. *S. aureus* possesses at least three members of the RpiR family of transcriptional regulators. Of the three RpiR homologs, RpiRc is a potential regulator of the pentose phosphate pathway, which also regulates RNAIII levels. RNAIII is the regulatory RNA of the *agr* quorum-sensing system that controls virulence determinant synthesis. The effect of RpiRc on RNAIII likely involves other regulators, as the regulators that bind the RNAIII promoter have been intensely studied. To determine which regulators might bridge the gap between RpiRc and RNAIII, *sarA*, *sigB*, *mgrA*, and *acnA* mutations were introduced into an *rpiRc* mutant background, and the effects on RNAIII were determined. Additionally, phenotypic and genotypic differences were examined in the single and double mutant strains, and the virulence of select strains was examined using two different murine infection models. The data suggest that RpiRc affects RNAIII transcription and the synthesis of virulence determinants in concert with $\sigma^B$, SarA, and the bacterial metabolic status to negatively affect virulence.**

*Staphylococcus aureus* is a major human pathogen causing a diverse range of infections, from superficial skin and wound infections to life-threatening diseases such as bacteremia, endocarditis, osteomyelitis, deep tissue abscesses, or pneumonia (1). The pathogenicity of *S. aureus* is due in part to its ability to produce a large number of virulence determinants, including secreted proteins (e.g., toxins and proteases), cell wall-associated proteins (e.g., protein A and fibronectin binding proteins), and extracellular polysaccharides (i.e., capsule and polysaccharide intercellular adhesion). During colonization and infection of a host, *S. aureus* must adapt to rapidly changing environmental and nutritional conditions by coordinating the transcription and translation of physiologic and virulence genes (2). To coordinately control these cellular processes, *S. aureus* has evolved or acquired a network of regulators such as the recently identified family of proteins, RpiR, that affect pentose phosphate pathway activity and virulence determinant synthesis (3).

Central to the regulatory network is the accessory gene regulator (Agr) system, a regulator of virulence determinant synthesis that responds to the bacterial population density (4). The *agr* locus consists of two divergent transcriptional units, RNAII and RNAIII, driven by the P2 and P3 promoters, respectively. RNAII comprises the *agrBDCA* operon, of which the *agrBD* gene products are involved in the synthesis, transport, and maturation of an autoinducing peptide (AIP). As the cell density increases, AIP accumulates in the extracellular milieu and when a threshold is achieved the two-component system AgrCA responds by activating transcription of both P2 and P3 promoters. The P3 promoter drives transcription of RNAIII, which is the regulatory effector of the Agr system and the mRNA that codes for delta-toxin. RNAIII reduces the expression of several cell surface proteins and activates the synthesis of many secreted proteins and capsule at the transcriptional and/or translational level.

The *Staphylococcus* accessory gene regulator, SarA, is a small DNA- and RNA-binding protein that originates from three overlapping transcripts initiated by individual promoters (5, 6). SarA also regulates transcription of many *S. aureus* virulence genes and functions in part by activating the *agr* promoters (5, 7–10). Regulation of virulence gene transcription by SarA involves direct binding of the protein to an AT-rich DNA motif (11); however, the molecular mechanisms remain unclear (10, 12–15). Both the *agr* and the *sarA* loci have been closely linked to the ability of *S. aureus* to invade and survive within a host, and mutants in either regulatory system are attenuated in virulence (16–22). In addition to SarA, there is another MarR protein family member called MgrA, which is a pleiotropic regulator that controls about 350 genes (23) affecting autolysis (24), antibiotic resistance (25), virulence determinants (26), and pathogenicity (27, 28). The activity of MgrA is mediated by a redox-switch and through interactions with other regulatory elements (23, 27, 29, 30). A number of other members of the MarR/SarA protein family such as Rot, SarS, SarR,

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**TABLE 1** Strains and plasmids used in this study

| Strain or plasmid | Description[a] | Source or reference |
|---|---|---|
| *S. aureus* strains | | |
| SA564 | *S. aureus* clinical isolate, wild type | 89 |
| BS687 | RN6734 *hldagrBDCA*::*ermB* | Bo Shopsin |
| CYL1050 | Newman *mgrA*::*cat* | 26 |
| UAMS-1 | UAMS-1 *sarA*::*kan* | 69 |
| HOM15 | COL *rsbUVW sigB*::*ermB* | 34 |
| SA-*rpiRc* | SA564 *rpiRc*::*tetM* | 3 |
| SA-*rpiRc_rpiRc* | SA564 *rpiRc*::*tetM*_pEC-*rpiRc*; *cis*-complemented SA-*rpiRc* mutant | This study |
| SA-*acnA* | SA564 *acnA*::*ermB* | 44 |
| SA-*acnA rpiRc* | SA564 *acnA*::*ermB rpiRc*::*tetM* | This study |
| SA-*agr* | SA564 *hldagrBDCA*::*ermB* | This study |
| SA-*agr rpiRc* | SA564 *hldagrBDCA*::*ermB rpiRc*::*tetM* | This study |
| SA-*mgrA* | SA564 *mgrA*::*cat* | This study |
| SA-*mgrA rpiRc* | SA564 *mgrA*::*cat rpiRc*::*tetM* | This study |
| SA-*sarA* | SA564 *sarA*::*kan* | This study |
| SA-*sarA rpiRc* | SA564 *sarA*::*kan rpiRc*::*tetM* | This study |
| SA-*sigB* | SA564 *rsbUVW sigB*::*ermB* | This study |
| SA-*sigB rpiRc* | SA564 *rsbUVW sigB*::*ermB rpiRc*::*tetM* | This study |
| Plasmids | | |
| pEC4 | pBluescript II KS(+) with *ermB* inserted into the ClaI site | 90 |
| pSB2035 | *E. coli-S. aureus* shuttle plasmid, luciferase reporter system of the *agr* P3 promoter; $Cm^r$ | 58 |

[a] $Cm^r$, chloramphenicol resistance.

SarU, and SarT have been identified as being involved in *agr* and *sarA* regulation (31).

The alternative sigma factor B ($\sigma^B$) regulates transcription of genes involved in the stress response that contribute to survival under unfavorable conditions like heat, oxidative, and antibiotic stresses (32–35). In addition, the *sigB* operon is linked to the complex network of virulence determinant regulation in *S. aureus* by altering transcription of *sarA* and RNAIII (36–41).

Inactivation of central metabolic pathways, such as the tricarboxylic acid (TCA) cycle, can affect virulence determinant biosynthesis by feedback and feedforward alteration of enzymatic activity or by altering the intracellular concentrations of metabolites to which metabolite-responsive regulators respond (42–46). Several metabolite-responsive regulators have been identified in *S. aureus* (e.g., CcpA [47, 48], CodY [49, 50], CcpE [51, 52], and Rex [53]) that link metabolism with virulence regulation. As examples, the carbon catabolite protein A (CcpA) responds to glucose-associated metabolic signals (54), whereas CodY responds to GTP and branched-chain amino acids (50, 55). As mentioned, we recently identified a putative metabolite-responsive family of regulators in *S. aureus*, RpiR, which contain a DNA-binding helix-turn-helix motif and a sugar isomerase binding domain (3). Inactivation of the *rpiRc* gene in *S. aureus* strain UAMS-1 dramatically increased the transcription and/or stability of RNAIII (3). To determine whether the effect of *rpiRc* inactivation on RNAIII levels required one or more of the known regulators of RNAIII transcription, we constructed *agr*, *sigB*, *sarA*, and *mgrA* regulatory mutants and an aconitase (*acnA*) mutant in strain SA564 and its isogenic *rpiRc* deletion mutant. The effects of these mutations on growth, cultivation pH profiles, and the transcription of RNAIII, *spa* (encoding protein A), *hla* (encoding alpha-hemolysin or alpha-toxin), and *capA* (encoding the initial capsule biosynthesis enzyme) were determined. In addition, the *in vivo* importance of RpiRc was assessed in chronic and acute murine infection models.

## MATERIALS AND METHODS

**Bacterial strains, materials, and growth conditions.** The strains used in this study are listed in Table 1. *S. aureus* strains were grown in tryptic soy broth (TSB) containing 0.25% glucose or on TSB plates containing 1.5% agar (TSA). Unless otherwise indicated, antibiotics were only used for strain construction and phenotypic selection at the following concentrations: tetracycline, 2.5 μg/ml; erythromycin, 2.5 μg/ml; kanamycin, 15 μg/ml; and chloramphenicol, 10 μg/ml. To inoculate cultures, bacteria from an overnight culture were inoculated 1:200 into TSB, cultured for 1.5 to 2 h, and harvested by centrifugation (5 min, 5,000 rpm), and the bacteria were diluted to an initial optical density at 600 nm ($OD_{600}$) of 0.05 in fresh medium. Bacterial cultures were grown with shaking at 225 rpm at 37°C, using a flask-to-medium ratio of 10:1.

**Phage transduction.** Mutants of strains SA564 and SA-*rpiR* were constructed by phage transduction. Briefly, donor strains were grown overnight in TSB at 37°C using a flask/medium ratio of 10:1 and with shaking at 225 rpm. The cultures were supplemented with $CaCl_2$ to a final concentration of 5 mM, and aliquots were infected with serial dilutions of transducing phage ϕ11, 80α, and/or ϕ85 and distributed on TSA plates using lysogeny broth medium with 0.6% agar containing 5 mM $CaCl_2$. After confluent lysis of the bacterial cells, the phage lysate was harvested and used to transfer mutations into recipient strains.

**RNA isolation and purification.** *S. aureus* strains were grown as described. Bacteria were harvested after 2, 4, 6, or 8 h cultivation by mixing with an equal volume of killing buffer (20 mM Tris/HCl [pH 7.5], 5 mM $MgCl_2$, 20 mM $NaN_3$) and centrifuged at 5,000 rpm at 4°C for 15 min. Total RNA was isolated from the pelleted bacteria using the FastRNA Pro Blue kit (Qbiogene) and was further purified using an RNeasy kit with on-column DNase treatment (Qiagen) according to the manufacturer's recommendations. Alternatively, bacteria were lysed in 1 ml of TRIzol reagent (Invitrogen Life Technologies, Karlsruhe, Germany) with 0.5 ml of zirconia-silica beads (0.1 mm in diameter) in a high-speed homogenizer (Savant Instruments, Farmingdale, NY). RNA was isolated as described in the instructions provided by the manufacturer of TRIzol. RNA concentrations were determined from the absorbance at 260 nm, and the RNA was examined by agarose gel electrophoresis to assess the quality.

**Complementation of *rpiRc* mutant.** Plasmid pEC4 containing an erythromycin resistance gene (*ermB*) and an ampicillin resistance gene (*bla*) was used to complement *rpiRc* mutants in *cis*. Briefly, the *rpiRc* gene from *S. aureus* strain SA564 was amplified by PCR using the primers MBH-467_SAV2315_compl-f and MBH-468_SAV2315_compl-r and was ligated into pEC4 after restriction with EcoRI and BamHI. The resulting plasmid, pEC4-*rpiRc*, was transformed into *Escherichia coli* strain DH5α and after isolation and verification electroporated into *S. aureus* strain RN4220. The plasmid was integrated into the chromosome via a single cross over and the region containing an intact *rpiRc* gene was phage transduced from RN4220 into the *rpiRc* mutant strains. Integration was verified by erythromycin selection and PCR using primer pairs ermB-int-+/ermB-int- and ermB-int-+/SAV2315_C_r (see Table S1 in the supplemental material).

**cDNA synthesis and quantitative real-time RT-PCR.** The Turbo DNA-free DNase treatment and removal kit (Ambion) was used according to the manufacturer's instructions to eliminate residual DNA contamination from total RNA preparations. RNA concentrations were determined from the absorbance at 260 nm, and the absence of chromosomal DNA was assessed by PCR and control reactions without reverse transcriptase (RT) during cDNA synthesis. DNA-free total RNA was synthesized to cDNA using the Bio-Rad iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad) according to the manufacturer's instructions.

Quantification of mRNA was performed using the Bio-Rad CFX Connect real-time PCR detection system (Bio-Rad) with 20-μl PCR mixtures containing Bio-Rad SsoAdvanced SybrGreen Supermix (Bio-Rad), gene-specific primer pairs listed in Table S1 in the supplemental material, and diluted cDNA template. No-template and no-RT controls were routinely analyzed with the test reactions. Reaction mixtures were incubated for 3 min at 94°C, followed by 40 cycles of 15 s at 94°C, 25 s at 60°C, and 20 s at 72°C, and single amplification products were verified by subsequent melting curve analysis. The increase in fluorescence was used to monitor amplification, and the threshold cycle ($C_T$) number was determined. Standard curves were generated for each primer pair to determine the amplification efficiency using serial dilutions of pooled cDNA samples and the Bio-Rad CFX Manager 2.1 software. These PCR efficiency values were applied in the subsequent calculation of relative mRNA levels for each gene. The transcriptional levels of target genes were normalized against the mRNA concentration of 16S rRNA and *gyrB* reference genes according to the $\Delta\Delta C_T$ method and scaled to the geometric mean of all samples. Samples were assayed at least in duplicate from three independent biological replicates.

**Northern blot analysis.** For Northern blot analysis, total RNA isolated from bacterial cultures was transferred to a positively charged membrane after electrophoresis as described previously (56). The intensities of the 23S and 16S rRNA bands stained by ethidium bromide were verified to be equivalent in all the samples before transfer. Digoxigenin (DIG)-labeled probes for the detection of specific transcripts were generated using a DIG-labeling PCR kit according to the manufacturer's instructions (Roche Biochemicals).

**Hemolytic activity.** The hemolytic activity of *S. aureus* strains was assessed qualitatively by growth on rabbit blood agar plates and evaluation of the lytic zones after incubation at 37°C for 24 h. Additionally, a microplate hemolytic assay using rabbit erythrocytes was performed. Twofold serial dilutions of 12-h culture supernatants in phosphate-buffered saline (PBS; pH 7.2) were mixed with an equal volume (100 μl) of 2% (vol/vol) washed rabbit erythrocytes in PBS in 96-well U-bottom microtiter plates. The plates were incubated at 37°C for 30 min, followed by further incubation at 4°C overnight. The microtiter plate was centrifuged at 600 × *g* for 5 min. The hemolytic activity titer was defined as the inverse of the last dilution that caused complete hemolysis.

**Western blot analyses.** To determine the effects of gene inactivation on protein A biosynthesis, the culture supernatants of *S. aureus* strains were collected, and optical density at 600 nm ($OD_{600}$)-adjusted aliquots were subjected to Western blot analysis as described previously (3).

**Capsule immunoblot assay.** The accumulation of capsule was quantified as described previously (26, 57). Briefly, equivalent numbers of bacteria were harvested by centrifugation, washed, and suspended in PBS and then treated with DNase I, lysostaphin, and proteinase K. After heat inactivation of the enzyme, serial dilutions of the crude extracts were assayed by immunoblotting.

**Luciferase activity assay.** RNAII transcription from the P3 promoter in *S. aureus* strains was examined by monitoring the luciferase activity produced by the *agr-lux* reporter plasmid pSB2035 (58). Bacteria were cultivated as described previously using medium containing 10 μg of chloramphenicol/ml. At the indicated time, bacterial aliquots (100 μl) were transferred in a black 96-well microtiter plate, and relative light unit readings were taken for 5 s at 37°C in a Wallac Victor2 1420 multilabel counter (Perkin-Elmer Life Sciences). A control sample of the wild type without plasmid was included to allow for background luminescence correction. For each sample, luciferase activity was normalized to the $OD_{600}$.

**Murine infection models.** All animal experiments were approved by the local State Review Boards of Saarland following the national guidelines for the ethical and humane treatment of animals.

Bacterial strains were grown overnight in TSB at 37°C, diluted to an initial $OD_{600}$ of 0.05 in fresh medium, and incubated at 37°C with a flask/medium ratio of 10:1 with 225-rpm aeration for 2 h. Bacteria from the exponential growth phase were harvested by centrifugation, washed, and suspended in sterile PBS. Bacterial suspensions were diluted to the desired $OD_{600}$ representing the correct infectious dose, which was validated by determining the colony formation on sheep blood agar plates. Female, 8- to 10-week-old C57/BL6N mice were used and maintained under specific-pathogen-free conditions.

For the systemic abscess model (47), 100-μl bacterial suspensions containing $10^7$ CFU were administered intravenously by retro-orbital injection into mice anesthetized by the intraperitoneal injection of 100 mg of ketamine hydrochloride (Pfizer, Berlin, Germany)/kg body weight and 10 mg of xylazine hydrochloride (Bayer, Leverkusen, Germany)/kg. At 4 days postinfection, the mice were sacrificed, and the kidneys and livers were removed. Portions of the liver and left kidney were fixed in 4% buffered formaldehyde, dehydrated, and embedded in paraffin. From each organ, 5-μm sections were stained with hematoxylin and eosin (H&E) according to standard protocols and analyzed by light microscopy. The remainders of each liver and right kidney were weight adjusted and homogenized in PBS, and serial dilutions of the homogenates were plated on sheep blood agar plates to enumerate the CFU.

In the lung infection model (59, 60), anesthetized mice were infected intranasally with $2.5 \times 10^8$ or $1 \times 10^8$ CFU of *S. aureus*. At 24 h postinfection, the animals were sacrificed, and a bronchoalveolar lavage (BAL) was performed before removal of the entire lung. To determine the bacterial load in the lung, serial dilutions of BAL and lung homogenates were plated on blood agar plates, and the CFU were determined. For statistical analysis, nonparametric Mann-Whitney tests were performed using Prism (GraphPad Software).

## RESULTS

**Inactivation of *rpiRc* pleiotropically affects transcription and accumulation of virulence determinants via the *agr* and *sarA* regulatory loci.** To determine whether the stimulatory effect of *rpiRc* inactivation on RNAIII levels was mediated by one of the established RNAIII regulators, double mutants were constructed (Table 1), and the effects on growth, virulence determinant transcription, and biosynthesis were assessed. Consistent with previous observations (3), inactivation of *rpiRc* in strain SA564 slightly altered growth in the postexponential growth phase (see Fig. S1 in the supplemental material). Interestingly, inactivation of *agr* and *sarA* in SA-*rpiRc* restored postexponential growth to wild-type levels. The restoration of growth is likely due to a redirection of carbon and energy from virulence determinant synthesis into bio-

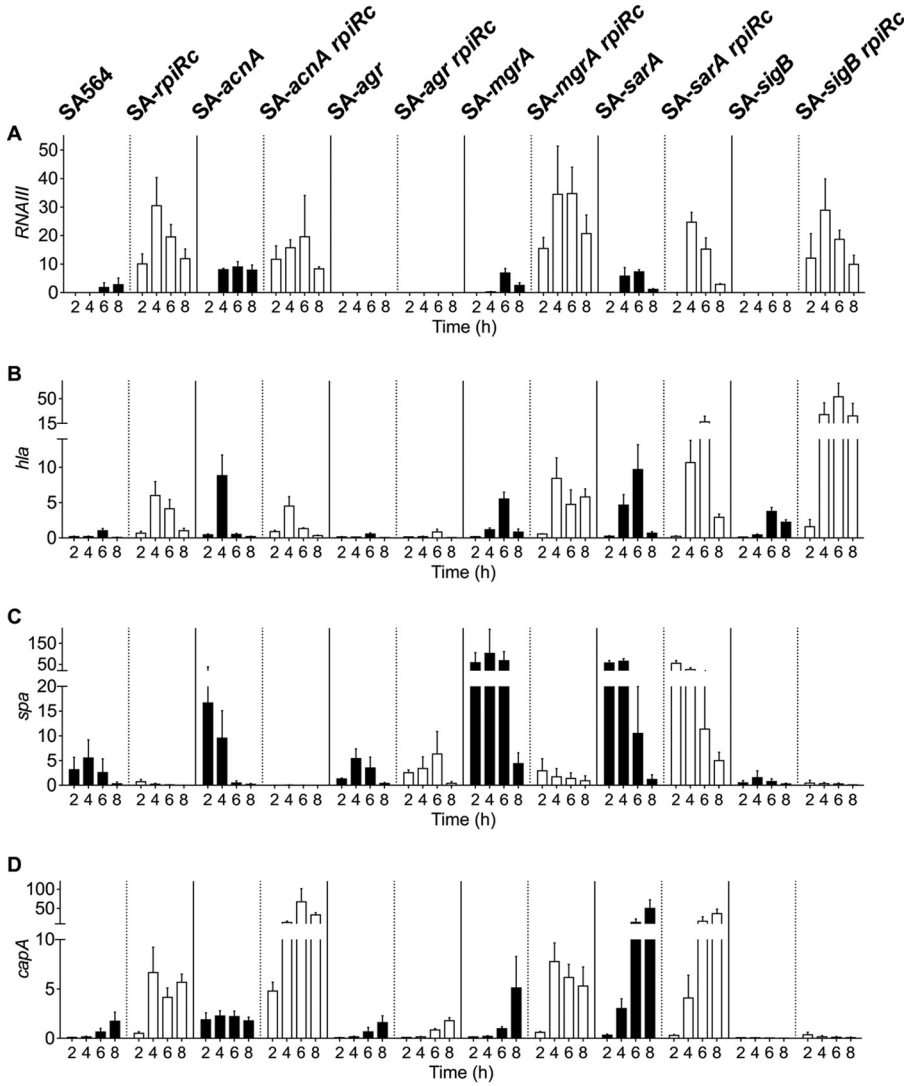


**FIG 1** Effect of *rpiRc* mutation on transcription of RNAIII (A) and genes encoding the virulence determinants alpha-toxin (B), protein A (C), and capsule (D) throughout the growth cycle. Quantitative real-time RT-PCR analyses of total RNA isolated from *S. aureus* strain SA564, isogenic single mutants of *acnA*, *agr*, *mgrA*, *sarA*, and *sigB* (black bars), and their respective *rpiRc* double mutants (white bars) cultivated for the indicated times were performed. The relative transcript levels of target genes were normalized to *gyrB* and 16S *rRNA*. Shown are the mean and standard deviation (SD) for at least three independent experiments performed in duplicate.

mass generation, but this requires further validation. In contrast to inactivation of *agr* and *sarA*, inactivation of *mgrA* and *sigB* in the SA-*rpiRc* mutant strain did not alter growth relative to the single mutant SA-*rpiRc* strain. Likewise, inactivation of *acnA* in strain SA-*rpiRc* further decreased the growth yield relative to *acnA* single mutant strain SA-*acnA*.

In agreement with previous observations (3), inactivation of *rpiRc* dramatically increased RNAIII levels in strain SA564, which is consistent with increased *hla* and *capA* mRNA levels and decreased *spa* mRNA (Fig. 1). These transcriptional changes were lost upon inactivation of either *agr* or *sarA*, suggesting that the effect of RpiRc on transcription is indirectly mediated through these global regulators. In contrast to the SA-*agr rpiRc* and SA-*sarA rpiRc* double-mutant strains, the high RNAIII levels in SA-*rpiRc* were independent of $\sigma^B$, MgrA, and TCA cycle activity. In contrast to previous observations reporting a negative regulatory effect of $\sigma^B$ on RNAIII transcription (37, 61), RNAIII transcription was also abolished in the SA-*sigB* mutant (Fig. 1A). Although the effects of RpiRc on RNAIII were independent of $\sigma^B$ and TCA cycle activity, a synergistic positive effect of RpiRc and $\sigma^B$ on *hla* mRNA levels was observed (Fig. 1B). Similarly, inactivation of *rpiRc* and *acnA* dramatically increased *capA* mRNA levels, most likely due to a redirection of carbon away from the TCA cycle and into capsule biosynthesis (62) (Fig. 1D).

To determine whether the transcriptional changes (Fig. 1) correlate with phenotypes, the accumulation of important virulence determinants was investigated. The hemolytic activity of SA-*rpiRc* was approximately 12 times greater than that of the wild-type strain SA564 and comparable to single and *rpiRc* double mutants of *acnA*, *mgrA*, or *sarA*. As expected, both *agr* mutants had no hemolytic activity, whereas the SA-*sigB rpiRc* mutant had increased hemolytic activity (Fig. 2A), which was consistent with the

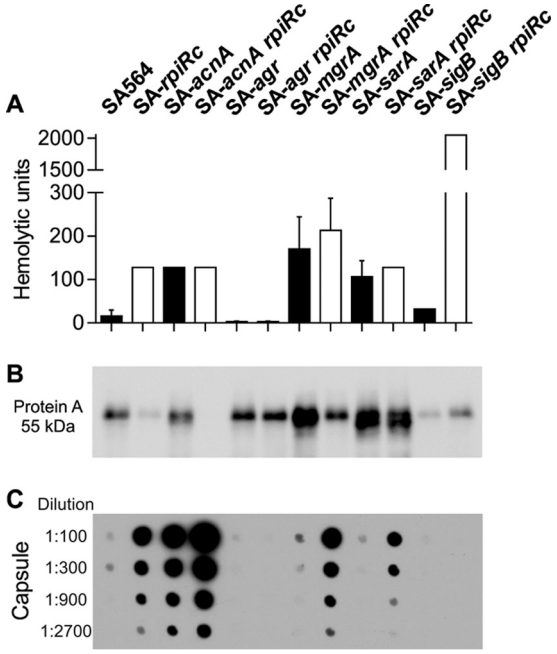


**FIG 2** Phenotypic characterization of virulence determinants in *S. aureus* regulatory mutants. *S. aureus* strain SA564, isogenic single mutants of *acnA*, *agr*, *mgrA*, *sarA*, and *sigB*, and their respective *rpiRc* double mutants were assayed for hemolytic activity (A), protein A accumulation (B), and capsule (C). In panel A, the means and standard deviations (SD) for at least three independent experiments are shown. In panel B, the Western blot is representative of at least three independent experiments. In panel C, the capsule immunoblot is representative of at least three independent experiments.

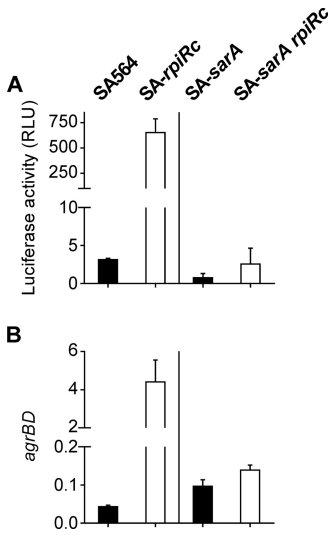


**FIG 3** Effect of the deletion of *rpiRc* and *sarA* on the *agr* quorum-sensing system in *S. aureus* strain SA564. *S. aureus* strain SA564 or *sarA* single mutant (■) and their isogenic *rpiRc* mutants (□) were cultivated for 2 h prior to harvest. (A) Luciferase activities (means ± the SD) of strains harboring the *agr* P3 promoter reporter plasmid pSB2035 from at least three independent experiments. (B) Total RNA was isolated from bacterial cells and subjected to quantitative real-time RT-PCR, and the relative transcript levels of *agrBD* were normalized to *gyrB* and 16S rRNA. Means and SD for at least three independent experiments performed in duplicate are shown.

transcriptional data for *hla* (Fig. 1B). In agreement with observations from *S. aureus* strain UAMS-1 (3), protein A was minimally synthesized in the *rpiRc* mutant of strain SA564, which also had greatly reduced *spa* mRNA levels. The negative effect of *rpiRc* inactivation on *spa* transcription or mRNA stability was abrogated by inactivation of the *sar* locus. In addition, *spa* transcription or mRNA stability and protein A accumulation were strongly decreased in the SA-*sigB* mutant and SA-*sigB rpiRc* double mutants (Fig. 1C and Fig. 2B). Similar to previous observations using strain UAMS-1 (3), inactivation of *rpiRc* tremendously increased the elaboration of capsule in SA564 (Fig. 2C). Importantly, these data demonstrate that the effects of *rpiRc* inactivation on virulence determinants are independent of the genetic background. In addition to RpiRc regulating capsule accumulation, capsule biosynthesis requires TCA cycle activity (62). Consistent with high *capA* mRNA levels in the exponential growth phase (Fig. 1D), the TCA cycle mutant strain SA-*acnA* showed increased capsule accumulation after 4 h of cultivation. An SA-*rpiRc acnA* double mutant had strongly increased capsule accumulation, indicating a synergistic repressive effect of RpiRc and TCA cycle activity on capsule biosynthesis. Inactivation of the global regulator *mgrA* had no effect on capsule accumulation in strain SA-*rpiRc*, whereas inactivation of *sarA* clearly reduced the amount of capsule detected, although it was still markedly higher than in the wild-type strain. No capsule was detected in the *agr* and *sigB* mutant pairs, highlighting the importance of these two regulators for capsule formation. Taken together, inactivation of *rpiRc* in *S. aureus* increased hemolytic activity, decreased protein A synthesis, and increased capsule synthesis, and these effects were partly mediated via the *agr* and *sarA* loci.

**Exponential growth phase transcription of the *agr* operon is increased in the *rpiRc* mutant in a SarA-dependent manner.** Inactivation of *rpiRc* in *S. aureus* strain SA564 increased RNAIII levels throughout the growth cycle. The exponential growth phase accumulation of RNAIII was abolished when *sarA* was inactivated, suggesting that SarA is required for the early accumulation of RNAIII in an *rpiRc* mutant (Fig. 1A). There are two possible explanations for the SarA-dependent increase in RNAIII levels; namely, an increase in transcription or a decrease in RNA turnover. To determine which of these possibilities was correct, a reporter plasmid (pSB2035) containing the *agr* P3 promoter fused to the *lux*-operon (58) was introduced into strains SA564, SA-*rpiRc*, SA-*sarA*, and SA-*sarA rpiRc*. Luciferase activity assays demonstrated that deletion of *rpiRc* increased transcription of RNAIII from the P3 promoter relative to the wild-type strain, indicating that the augmented RNAIII transcript levels were due to increased transcription rather than reduced RNAIII degradation. Consistent with the quantitative PCR (qPCR) data, the SA-*sarA rpiRc* double mutant showed markedly reduced luciferase activity, confirming that the presence of *sarA* is required for high *agr* promoter activity in an *rpiRc* mutant background (Fig. 3A). In order to examine if the effect of RpiRc and SarA on the Agr system extends to the P2 promoter driven transcription of RNAII, quantitative PCR (qPCR) on the *agrBD* genes was performed. Similar to RNAIII transcription and luciferase activity data, the *agrBD* transcript levels were elevated in the SA-*rpiRc* mutant compared to the wild-type and the SA-*sarA* and SA-*sarA rpiRc* mutant strains (Fig. 3B). In total, these data demonstrate that SarA is required for increased transcription of the *agr* quorum-sensing system in an *S. aureus rpiRc* mutant during the exponential growth phase.

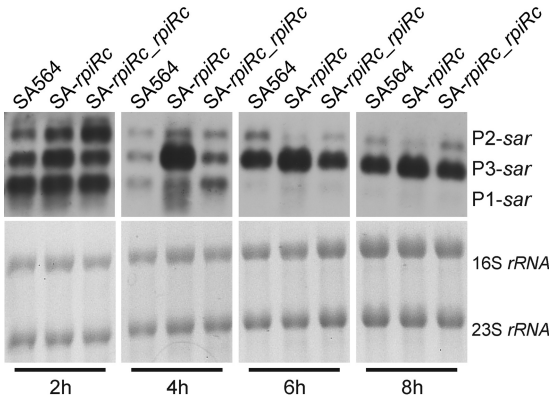


**FIG 4** Positive effect of *rpiRc* inactivation on transcript levels of *sarA*. Northern blot analysis of *sar* transcripts from *S. aureus* strain SA564, the *rpiRc* deletion mutant (SA-*rpiRc*), and the *rpiRc* mutant carrying the *cis*-integrated plasmid pEC4-*rpiRc* for complementation (SA-*rpiRc_rpiRc*) cultivated for the indicated times was performed. The *sar* locus originates from three different promoters (P1, P3, and P2) resulting in transcripts that differ in size (0.56, 0.8, and 1.2 kb, respectively). The blot is representative of at least two independent experiments.

**Inactivation of *rpiRc* increases *sarA* transcription.** Transcription of RNAIII and virulence determinant synthesis were strongly affected by RpiRc in an *agr*- and *sarA*-dependent manner. Based on these data, it was reasonable to hypothesize that RpiRc may exert its regulatory effects by influencing *sarA* transcription. The *sar* locus is composed of three overlapping transcripts (termed *sarA*, *sarC*, and *sarB*) that initiate from the promoters P1, P3, and P2, respectively. All three transcripts encompass the major open reading frame, *sarA*, which codes for the SarA protein (5). Northern blot analysis using a *sarA* specific probe was performed on total RNA isolated from strains SA564, SA-*rpiRc*, and the complemented mutant SA-*rpiRc_rpiRc* (Fig. 4). In the wild-type strain, *sarA*, *sarC*, and *sarB* transcripts were present during the exponential growth phase; however, the intensity of the *sarA* and *sarB* transcripts decreased over time. This was consistent with previous observations that *sarC* transcripts increased during the postexponential growth phase (39, 41). In strain SA-*rpiRc* the transcription of *sarC* was markedly increased compared to the wild-type strain SA564. The latter observation was interesting because the *sarC* transcript initiates from the $\sigma^B$-dependent P3 promoter. Lastly, complementation of the *rpiRc* mutation restored *sar* mRNA levels to that of the wild type.

**Transcription of *rpiRc* is constant throughout the growth cycle.** Members of the RpiR protein family are metabolite-responsive transcriptional regulators that consist of an N-terminal helix-turn-helix DNA binding motif and a C-terminal sugar isomerase binding domain (63, 64). RpiR regulators can respond to metabolic stimuli and trigger appropriate cellular responses by altering transcription of their respective target genes. In other words, control of RpiR regulatory activity likely occurs at the posttranslational level and is mediated by the concentration of a particular metabolite(s). Although RpiR activity likely occurs posttranslationally, it is possible that additional layers of regulation may occur that increase or decrease the intracellular copy numbers of RpiRc. To determine whether transcription of *rpiRc* is temporally modulated in *S. aureus*, qPCR was performed throughout the growth cycle (see Fig. S2 in the supplemental material). In the wild-type strain SA564, mRNA levels of *rpiRc* remained relatively constant during growth. Additionally, *rpiRc* mRNA profiles were similar to those of the wild-type strain in the *agr*, *sarA*, and *sigB* mutants. In contrast, the multiple gene regulator A, MgrA, exerted a small positive effect on *rpiRc* mRNA levels (an ~2.5-fold difference). Interestingly, inactivation of *acnA* increased the level of *rpiRc* mRNA in the exponential growth phase (2.9-fold higher) and yet decreased *rpiRc* mRNA (2.3-fold lower) in the postexponential growth phase (see Fig. S2 in the supplemental material). In summary, *rpiRc* mRNA levels are relatively constant during growth and are minimally affected by the most well-studied virulence regulators in *S. aureus*, indicating that the mode of regulation of RpiRc likely depends on the metabolic state of *S. aureus*.

**RpiRc attenuates staphylococcal pneumonia and abscess formation.** *S. aureus* strains lacking *rpiRc* have major alterations in RNAIII levels that correlated with phenotypic changes of a diverse set of virulence factors (Fig. 1 and 2). These observations suggest that RpiRc may be important in the host-pathogen interaction and affect the pathogenicity of *S. aureus*. To test this suggestion, the virulence of *S. aureus* strain SA564, its isogenic *rpiRc* mutant, and a complemented *rpiRc* mutant were assessed in a murine model of acute pneumonia (Fig. 5A). C57BL/6N mice were intranasally infected with an inoculation dose of $2.5 \times 10^8$ CFU per mouse, and survival was assessed for 24 h. All animals infected with SA-*rpiRc* succumbed to the infection within 18 h postinoculation, whereas all mice infected with either the wild type or the *rpiRc* complemented SA-*rpiRc* mutant were alive after 24 h (Fig. 5A). To determine whether there were differences in the lung bacterial burden, C57BL/6N mice were intranasally infected with a lower infectious dose ($10^8$ CFU), the animals were sacrificed after 24 h, the BAL fluids and lung tissues were collected, and viable bacteria enumerated. As expected, the bacterial loads in mice infected with SA-*rpiRc* were ~1.3-log higher in both BAL fluid (Fig. 5B) and lung tissue (Fig. 5C) relative to animals infected with strain SA564. The increased bacterial burden of the SA-*rpiRc* mutant strain in the murine lung is consistent with an increased severity of the infection. Importantly, complementation of the mutant restored wild-type CFU levels, confirming that the observed differences in the mutant were attributable to the absence of *rpiRc*. Because some of the effects of *rpiRc* inactivation are dependent upon SarA (Fig. 1, 3, and 4), we sought to determine whether the increased virulence of SA-*rpiRc* (Fig. 5) was influenced by SarA. To test this hypothesis, mice were infected with the SA-*sarA rpiRc* double mutant, SA-*sarA*, or the wild-type strain SA564, and survival was assessed. The bacterial loads of the *sarA* single and double mutants in lung tissues and BAL samples ranged between both the SA564 wild type and the *rpiRc* mutant without significant differences, suggesting that SarA is required for the enhanced infectivity of the SA-*rpiRc* mutant in this model.

To determine whether this difference in virulence was restricted to the lungs or whether this was a more general phenomenon, a mouse systemic abscess formation model was used to assess the bacterial ability to establish abscesses in the liver and kidneys. In this model, mice were retro-orbitally infected with $10^7$ CFU of *S. aureus* strain SA564 or its derivatives, and the livers and kidneys were removed at 4 days postinfection for examination of abscess formation and enumeration of the bacterial burden. Histopathology revealed similar abscess lesions within the liver tissue obtained from wild type- and SA-*rpiRc*-challenged mice (Fig. 6A); however, the number of abscesses (Fig. 6B) and the bacterial bur-

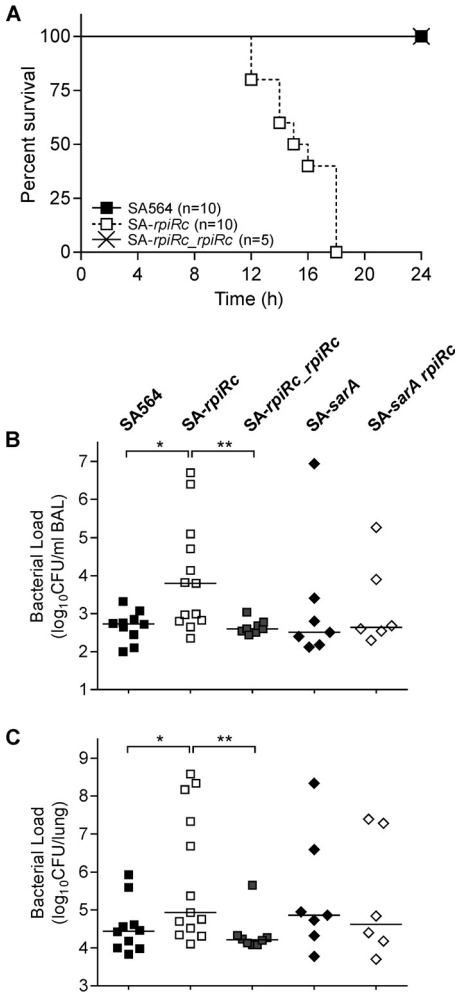


**FIG 5** Inactivation of *rpiRc* results in reduced survival (A) and increased bacterial burden (B and C) of *S. aureus* in a murine pneumonia model. Wild-type *S. aureus* strain SA564, the *rpiRc* deletion mutant (SA-*rpiRc*), the *rpiRc* mutant carrying the *cis*-integrated plasmid pEC4-*rpiRc* for complementation (SA-*rpiRc_rpiRc*), and the *sarA* single (SA-*sarA*) and *rpiRc* double (SA-*sarA rpiRc*) mutants were grown to exponential growth phase (2 h), washed, and intranasally administered to 8- to 9-week-old C57/BL6N mice. (A) The survival of mice using an infectious dose of $2.5 \times 10^8$ CFU was monitored. (B and C) After infection with $10^8$ CFU, the bacterial loads in BAL samples (B) and lung tissue homogenates (C) were determined at 24 h postinfection. Each symbol represents an individual animal, and the mean values per group are depicted by horizontal lines. For statistical analysis, nonparametric Mann-Whitney tests were performed (*, $P < 0.05$; **, $P < 0.01$).

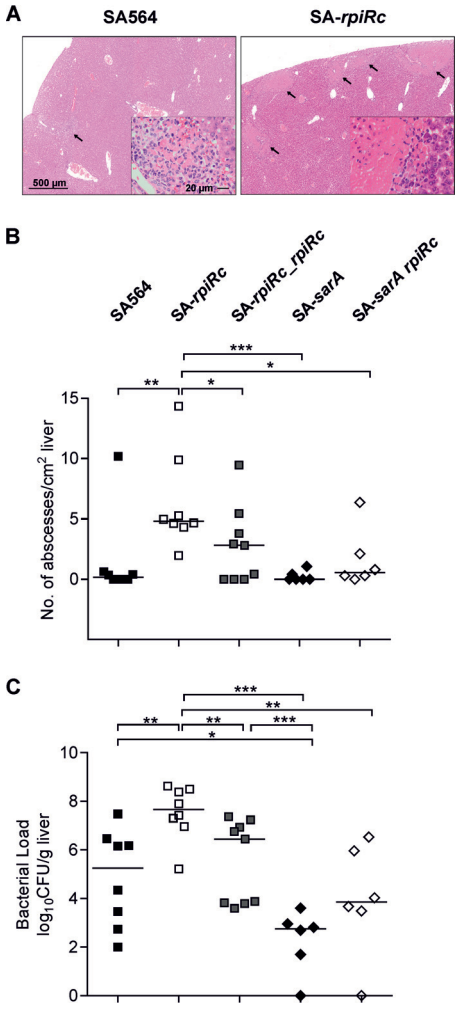


**FIG 6** Inactivation of *rpiRc* results in more abscesses (A and B) and higher bacterial burdens (C) in a murine abscess formation model. *S. aureus* wild-type strain SA564, the *rpiRc* deletion mutant (SA-*rpiRc*), the *rpiRc* mutant carrying the *cis*-integrated complementation plasmid pEC4-*rpiRc* (SA-*rpiRc_rpiRc*), and the *sarA* single (SA-*sarA*) and *rpiRc* double (SA-*sarA rpiRc*) mutants were grown to exponential growth phase (2 h) and washed, and $10^7$ CFU were administered via retroorbital injection to C57BL/6N mice. At 4 days postinfection, the mice were euthanized, and the livers were removed. Representative H&E staining of liver sections from the wild type and the *rpiRc* mutant showing abscess lesions (arrows) is shown. In panel A, the magnification bar represents 500 μm (20 μm in each inset). (B) The number of abscesses was enumerated and normalized to the observed tissue area. (C) Bacterial loads in homogenized liver. Each symbol represents an individual animal, and the mean values per group are depicted by horizontal lines. For statistical analysis, nonparametric Mann-Whitney tests were performed (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

den (Fig. 6C) were significantly elevated in mice infected with strain SA-*rpiRc* relative to the mouse group challenged with the parental strain SA564. In contrast to the acute pneumonia model, inactivation of *sarA* resulted in significant reductions in the bacterial burden and abscesses in liver tissue compared to SA564, SA-*rpiRc*, and SA-*rpiRc_rpiRc* (Fig. 6). The bacterial burden in organs from mice infected with the SA-*sarA rpiRc* double mutant was significantly reduced compared to that seen with SA-*rpiRc*-infected mice and similar to that seen with SA-*sarA*-infected mice, confirming the prominent role of SarA in murine infection (20–22). Similarly, bacterial burdens were increased in the kidneys of mice infected with strain SA-*rpiRc* relative to the isogenic parental strain SA564 (data not shown). Taken together, the RpiRc regulator serves as an important negative modulator of pathogenicity in *S. aureus* in two different murine infection models, and this was partially dependent on SarA.

## DISCUSSION

During aerobic planktonic growth, *S. aureus* genes encoding cell surface proteins are upregulated in the exponential growth phase and downregulated in the postexponential growth phase, whereas genes encoding secreted toxins and capsule are upregulated in the postexponential growth phase (4, 31). This growth-phase-depen-

dent switch between cell-associated proteins and secreted proteins coincides with derepression of the TCA cycle (43, 44, 65) and activation of the Agr quorum-sensing system (4). Inactivation of *rpiRc* greatly increased the transcription of RNAIII (Fig. 1A and Fig. 3A), suggesting that RpiRc can function as a metabolite-responsive modulator of the *agr* quorum-sensing system. Not only did *rpiRc* inactivation increase RNAIII transcription, but it also increased RNAII synthesis (Fig. 3B), which suggests that RpiRc suppresses quorum-sensing. This suppression of quorum sensing is likely indirect, requiring the action of SarA in the exponential growth phase. In contrast, transcriptional regulation of *hla* was more complicated. Deletion of *rpiRc* and *sigB*, also a negative effector of alpha-toxin (36, 38, 40), resulted in a synergistic increase in *hla* mRNA and increased hemolysis of erythrocytes, suggesting a regulatory linkage between RpiRc and $\sigma^B$. Interestingly, inactivation of *sarA* increased *hla* mRNA levels and hemolytic activity relative to the wild-type strain SA564; however, a *rpiRc sarA* double mutant had an only slightly increased *hla* mRNA level but similar hemolytic activity to the wild-type strain. This could be due to SarA having both an activating (66–68) and a repressing (69–71) influence on *hla* transcription, depending on the regulatory configuration of the strain genetic background (i.e., the presence of $\sigma^B$ or SarS [61, 69]).

In contrast to secreted toxins, the synthesis of cell surface-associated proteins (e.g., protein A) is greatest at low cell densities and is repressed by RNAIII (4). Specifically, RNAIII negatively affects protein A at the posttranscriptional level by binding *spa* mRNA and facilitating RNase III-mediated degradation (72, 73). As with our previous observations (3), protein A accumulation was nearly abolished in the *rpiRc* mutant throughout growth. As expected, *acnA* and *mgrA* inactivation increased *spa* mRNA levels relative to the wild-type strain (26, 29); however, *spa* mRNA levels were markedly reduced in the *acnA* and *mgrA* mutant strains when *rpiRc* was deleted. This is interesting because both MgrA and TCA cycle activity suppress *spa* transcription, suggesting that both MgrA and TCA cycle activity have lesser roles in influencing *spa* transcription than does RpiRc. Conversely, the SA-*sarA rpiRc* double mutant maintained elevated *spa* levels, signifying that RpiRc is dependent on the presence of SarA to exert its role on protein A synthesis. Lastly, the transcription and synthesis of protein A required $\sigma^B$ and inactivation of *rpiRc* could not overcome the absence of $\sigma^B$. Taken together, it seems likely the decrease in protein A synthesis in strain SA-*rpiRc* is due to an increase in $\sigma^B$-dependent transcription of the *sar* locus (Fig. 4).

Capsule protects *S. aureus* and enhances virulence by impeding C3 complement binding and phagocytosis (74). Typically, capsule is synthesized in the postexponential growth phase, and this requires TCA cycle activity (62). As expected, *capA* mRNA, which encodes the first enzyme for capsule biosynthesis, increased over time in wild-type strain SA564. Interestingly, inactivation of *acnA* resulted in very high *capA* mRNA levels throughout the growth cycle (Fig. 1D) (36). In addition, inactivation of the TCA cycle did not decrease capsule accumulation but actually increased it. This contrast between the present study and our previous work is likely due to differences in cultivation times; specifically, in the previous study, overnight cultures were used for capsule analyses, whereas in the present study bacteria were harvested after 4 h of incubation when preferred carbon sources are still available. Consistent with this suggestion, after 18 h of cultivation, capsule accumulation was nominal in the *acnA* mutant (data not shown). Similar to *acnA* inactivation, mutation of *rpiRc* greatly increased *capA* mRNA levels, and this effect was synergistic in the SA-*acnA rpiRc* double mutant. Interestingly, capsule accumulation was still elevated in strain SA-*acnA rpiRc* after 18 h of growth (data not shown), indicating that metabolic alterations due to disruption of *rpiRc* can overcome the TCA cycle-dependent requirements for capsule biosynthesis. Despite no major differences in *capA* mRNA levels between single and double mutants of *mgrA* or *sarA* and *rpiRc*, the accumulation of capsule was strongly enhanced in the presence of an *rpiRc* mutation, further suggesting that RpiRc exerts some of its effects independent of transcriptional regulation. In contrast, the negative effect of *sigB* inactivation (36, 75) abolished the transcription and synthesis of capsule in *S. aureus*, irrespective of the presence of RpiRc. Taken together, RpiRc-dependent effects on RNAIII and virulence determinants are linked to $\sigma^B$, SarA, and the bacterial metabolic status.

Inactivation of *rpiRc* strongly altered virulence determinant regulation; hence, it was reasonable to hypothesize that RpiRc alters the host-pathogen interaction and disease progression. To assess the effect of *rpiRc* inactivation on *S. aureus* virulence, two murine infection models were used: a pulmonary infection model and a systemic abscess model. These models were chosen because they mimic *S. aureus* infections that are frequently associated with difficult to treat complications in humans (76–79) and because RNAIII-regulated virulence determinants are known to be important in both models (77, 80–82). For example, the RNAIII-regulated alpha-hemolysin is important for respiratory disease progression by functioning as a cytotoxic factor that promotes pore formation and facilitates *S. aureus* entry into the peripheral blood system (59, 83, 84). In the murine model of pneumonia, 100% of the mice infected with strain SA-*rpiRc* succumbed to the infection within 24 h, whereas all mice infected with the wild-type strain survived, demonstrating the importance of RpiRc in the infection process. This is consistent with higher bacterial loads in the BAL samples and lung homogenates of mice infected with the *rpiRc* mutant strain than in those of mice infected with the wild-type strain SA564. In the murine abscess model, systemically applied *S. aureus* can disseminate within the host and form abscesses at almost any anatomical site (77). In this infection model, alpha-hemolysin (77), protein A (80), and capsule (85, 86) are important bacterial determinants of abscess formation. Similar to the pneumonia model, inactivation of *rpiRc* increased the bacterial burden and the number of abscesses in visceral organs, demonstrating that the importance of RpiRc to pathogenicity is independent of the animal model. The *in vitro* importance of SarA in mediating some aspects of RpiRc regulation of virulence determinants led us to examine whether SarA mediates the *in vivo* effects of *rpiRc* inactivation using both infection models. The virulence levels of strains SA-*rpiRc* and SA-*rpiRc sarA* were similar (Fig. 5 and 6), suggesting that the presence of SarA is required for RpiRc to exert its full function as an attenuator of virulence. The metabolite-responsive regulators CodY (87) and CcpE (51, 88) have similar negative effects on pathogenicity. In contrast, inactivation of *ccpA* decreased the bacterial load in a murine infection model (47). This is likely due to CcpA affecting transcription of virulence genes in *S. aureus*, in particular the exotoxin alpha-toxin gene (48).

**Conclusion.** The RpiR family of metabolite-responsive regulators functions to link central metabolism to virulence determinant biosynthesis in *S. aureus* (3). This linkage is critical because it connects the availability of the central metabolism generated bio-

synthetic intermediates (e.g., α-ketoglutarate and glucose-6-phosphate) with the synthesis of virulence determinants. Without biosynthetic intermediates, synthesis of biosynthetic precursors (e.g., amino acids and amino sugars) will be impaired, which will prevent the polymerizing and assembly reactions from producing virulence determinants and other cellular components. In the present study, we determined that the *agr* and *sar* loci and $\sigma^B$ function with RpiRc to modulate transcription and synthesis of several critical *S. aureus* virulence determinants. The significance of RpiRc was demonstrated in an acute pneumonia model and a systemic abscess formation model, where inactivation of *rpiRc* markedly increased mortality and the bacterial burden in mice. These data demonstrate that RpiRc functions to repress virulence determinant biosynthesis, which attenuates virulence in this clinically important pathogen. The effects of RpiRc are likely mediated through its ability to bind metabolites; however, this requires additional testing.

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We declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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