

Differential Expression and Roles of *Staphylococcus aureus* Virulence Determinants during Colonization and Disease

Amy Jenkins,^a Binh An Diep,^{b,c} Thuy T. Mai,^b Nhung H. Vo,^b Paul Warrener,^a Joann Suzich,^a C. Kendall Stover,^a Bret R. Sellman^a

Department of Infectious Disease, MedImmune, Gaithersburg, Maryland, USA^a; Department of Medicine, Division of Infectious Diseases, University of California, San Francisco, San Francisco, California, USA^b; Division of Infectious Diseases and Vaccinology, School of Public Health, University of California Berkeley, California, USA^c

ABSTRACT *Staphylococcus aureus* is a Gram-positive, commensal bacterium known to asymptotically colonize the human skin, nares, and gastrointestinal tract. Colonized individuals are at increased risk for developing *S. aureus* infections, which range from mild skin and soft tissue infections to more severe diseases, such as endocarditis, bacteremia, sepsis, and osteomyelitis. Different virulence factors are required for *S. aureus* to infect different body sites. In this study, virulence gene expression was analyzed in two *S. aureus* isolates during nasal colonization, bacteremia and in the heart during sepsis. These models were chosen to represent the stepwise progression of *S. aureus* from an asymptomatic colonizer to an invasive pathogen. Expression of 23 putative *S. aureus* virulence determinants, representing protein and carbohydrate adhesins, secreted toxins, and proteins involved in metal cation acquisition and immune evasion were analyzed. Consistent upregulation of *sdrC*, *fnbA*, *fhuD*, *sstD*, and *hla* was observed in the shift between colonization and invasive pathogen, suggesting a prominent role for these genes in staphylococcal pathogenesis. Finally, gene expression data were correlated to the roles of the genes in pathogenesis by using knockout mutants in the animal models. These results provide insights into how *S. aureus* modifies virulence gene expression between commensal and invasive pathogens.

IMPORTANCE Many bacteria, such as *Staphylococcus aureus*, asymptotically colonize human skin and nasal passages but can also cause invasive diseases, such as bacteremia, pneumonia, sepsis, and osteomyelitis. The goal of this study was to analyze differences in the expression of selected *S. aureus* genes during a commensal lifestyle and as an invasive pathogen to gain insight into the commensal-to-pathogen transition and how a bacterial pathogen adapts to different environments within a host (e.g., from nasal colonization to invasive pathogen). The gene expression data were also used to select genes for which to construct knockout mutants to assess the role of several proteins in nasal colonization and lethal bacteremia. These results not only provide insight into the factors involved in *S. aureus* disease pathogenesis but also provide potential therapeutic targets.

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Address correspondence to Bret R. Sellman, sellmanb@medimmune.com.

Staphylococcus aureus is a Gram-positive bacterium that causes both community- and hospital-acquired infections (1). Approximately 20 to 30% of the population is reported to be permanent carriers in the nose and 60% are intermittent carriers (2). Nasally colonized individuals are at increased risk for developing an infection with their colonizing isolate (2, 3). *S. aureus* infections range from mild skin and soft tissue infections to more severe infections, including bacteremia, sepsis, and osteomyelitis (1).

Differential gene expression has been described for many bacteria under various growth conditions or during infection (4–8). Due to the difficulty in studying bacterial gene expression *in vivo*, many studies have been performed in *in vitro* systems meant to mimic the host environment, including iron and nutrient limitation, conditions of low oxygen, biofilm versus planktonic bacterial growth, and following exposure to blood *in vitro* (4–8). These studies have provided valuable information about genes involved in the bacterial response to particular host environments when mimicked *in vitro*; however, the *in vivo* setting is far more complex.

In addition to *in vitro* studies, *S. aureus* gene expression has been characterized *in vivo*. Expression of a limited number of genes has been investigated in both device-related and wound infections (9–11), during skin infections (12, 13), human nasal colonization (14), kidney infection in mice (13), and in a cotton rat nasal colonization model (15). In these studies, transcript levels *in vivo* were compared with transcript levels measured under *in vitro* growth conditions. A comparison of gene expression in *S. aureus* between disease states has not been reported. The goal of this study was to compare the expression profile of 23 putative *S. aureus* virulence determinants in two clinical isolates during three stages of infection: nasal colonization, early bacteremia, and infected heart tissue (thromboembolic lesions) in a sepsis model. These models were chosen to represent the stepwise progression of individual *S. aureus* strains from a commensal (asymptomatic nasal colonization) to a pathogen (from early bacteremia and finally to an invasive infection during sepsis). Our results revealed upregulation of *sdrC*, *fnbA*, *fhuD*, *sstD*, and *hla* in *S. aureus* as an invasive pathogen compared to a commensal. These findings sug-

gest roles for these proteins in the progression of *S. aureus* infection and support further investigation of these factors as therapeutic targets.

RESULTS

Quantitative real-time PCR (qRT-PCR) was utilized to determine how *S. aureus* gene expression responds to environmental changes in the host, using different animal models (cotton rat nasal colonization and murine bacteremia and lethal sepsis). Cotton rat nasal colonization was chosen because it is well characterized and closely resembles chronic human nasal colonization. Alternatively, *S. aureus* is either cleared or requires antibiotics for chronic murine nasal colonization (16–20). Murine bacteremia and sepsis models were used because these models are not established for cotton rats. Four *S. aureus* gene classes were chosen for analysis: protein and carbohydrate adhesins, metal cation transporters, exotoxins, and immune evasion proteins (see Table S1 in the supplemental material).

One concern when running qRT-PCR on samples purified from an *in vivo* setting is that contaminants could copurify with the cDNA that inhibit the PCR. To address this concern, cDNA samples from each of the different infection sites (cotton rat nose and mouse blood and heart) were spiked into a control PCR assay specific for *Pseudomonas aeruginosa pcrV* on a plasmid. All of the samples amplified the *pcrV* gene with similar efficiency, indicating that none of the *in vivo* purified cDNA samples regardless of infection site adversely affected PCR efficiency (see Fig. S1 in the supplemental material).

Nasal colonization. Experiments were performed with two *S. aureus* clinical isolates, SF8300 (CC8; USA300 methicillin-resistant *S. aureus* [MRSA]) and ARC633 (CC15; methicillin-susceptible *S. aureus* [MSSA]) obtained from a skin abscess and from a nasal carrier, respectively. To establish a baseline condition (commensal), transcript levels were measured during cotton rat nasal colonization. To avoid the pitfalls of comparing expression data to data for expression levels under an arbitrary *in vitro* growth condition, expression levels were represented as the fold change from the minimum and maximum *in vitro* expression levels (a description of the *in vitro* growth conditions that resulted in these expression levels can be found in Table S2 in the supplemental material). 16S rRNA was selected as an internal control for normalization because its expression varied less than 2-fold for both SF8300 and ARC633 under various *in vitro* growth conditions (e.g., early-log, mid-log, late-log, and stationary phases in trypticase soy broth [TSB], RPMI, and human serum) (see Fig. S2 in the supplemental material). As such, a change in gene expression was defined as a greater-than-2-fold increase or decrease in transcript levels. During nasal colonization, transcript levels of four adhesin genes (*clfB*, *sdrC*, *sdrD*, and *tarK*) increased over the highest observed *in vitro* expression levels in both strains. An additional four genes exhibited increased expression over the highest *in vitro* expression levels in one strain (*clfA*, *icaB*, *efb*, and *sasG*) (Fig. 1). Increased *clfB*, *sdrC*, *sdrD*, *sasG*, and *tarK* transcript levels support previous results implicating their corresponding proteins in nasal colonization (14, 15, 21–23). Metal ions are limiting *in vivo*; therefore, one would expect metal cation transporters to be upregulated (24, 25). As expected, most metal cation transporter genes were upregulated relative to the minimal *in vitro* expression level (Fig. 2). The genes encoding SpA and Sbi showed increased expression over their lowest *in vitro* expression level and were un-

changed or decreased from the highest *in vitro* expression level. Toxin gene expression decreased compared to the lowest *in vitro* expression condition (Fig. 3). Of note, ARC633 does not encode *lukF-PV*.

Nasal colonization versus early bacteremia. Gene expression was then assessed in the blood of bacteremic mice 1 h postinfection (via intravenous [i.v.] injection). Transcript levels were compared to those observed in nasal colonization to determine which genes the bacteria modulate upon exposure to the bloodstream. Of the adhesins, only *fnbA* increased expression in both strains compared to nasal colonization, whereas *sdrC* and *icaB* showed increased expression in SF8300 and *sasA*, *tagO*, *efb*, and *ebpS* showed increased expression in ARC633 upon exposure to the bloodstream. Three metal cation acquisition genes had increased expression (*mntC*, *fhuD*, and *sstD*) compared to nasal colonization in ARC633. The *hla* gene was upregulated in both strains, whereas *lukF-PV* (SF8300) and *sbi* and *spa* (ARC633) were upregulated in one strain (Fig. 4). Taken together, these results indicate that individual *S. aureus* isolates respond differently to environmental change and may use different virulence determinants during the same type of infection.

Transition from early bacteremia to heart lesions. Transcript levels were measured in bacteria present in saline-perfused hearts 14 h postchallenge (i.v.) and compared to those in blood 1 h postinfection. The hearts were flushed with saline to ensure transcript levels were measured from bacteria in the heart tissue and not in residual blood. It should be noted that, similar to previous reports (26), we did observe histological evidence of thromboembolic lesions in the hearts of infected mice (data not shown). The comparisons performed here were undertaken to highlight genes regulated upon transition to the thromboembolic heart lesions characteristic of *S. aureus* sepsis (26), relative to the initial stages of bacteremia. Six adhesin genes (*clfA*, *sdrC*, *sasF*, *tagO*, *fnbA*, and *tarK*) displayed increased expression in both strains, and *sasA* (SF8300) and *icaB* (ARC633) showed increased expression in one strain in thromboembolic lesions relative to early bacteremic blood. Of these, *clfA*, *tagO*, and *fnbA* have been implicated in establishing organ colonization (26–29). Four cation transport genes (*mntC*, *isdB*, *fhuD*, and *isdA*) exhibited increased expression in both strains relative to early bacteremic blood, whereas *isdH* and *sstD* showed increased expression in ARC633. Upon seeding of the heart, *hla*, *sbi*, and *lukF-PV* all showed increased expression in SF8300, while *hla* and *sbi* remained constant in ARC633 (Fig. 5). In general, these results suggest that *S. aureus* utilizes multiple adhesins in the process of bloodstream escape and organ invasion. Additionally, upregulation of the metal cation transporters points to the iron-limiting nature of the heart, while exotoxins and immune evasion genes are likely utilized to escape the bloodstream and host defenses.

Isogenic mutant analysis. To compare gene expression analysis with a gene's role in pathogenesis, isogenic deletion mutants were constructed in SF8300. The genes (*clfA*, *clfB*, *sdrC*, *isdH*, *isdB*, *hla*, and *spa*) were chosen to represent a variety of expression profiles in the three models. The adhesins *clfA* and *sdrC* were chosen based on their consistent upregulation during infection. *clfB*, *isdH*, and *isdB* were chosen based on their upregulation in the nasal colonization model and in the transition from bloodstream to invasive pathogen (*isdB*). The remaining genes (*hla* and *spa*) showed substantially increased expression in two models and are known to play a role in *S. aureus* pathogenesis. Cotton rats were

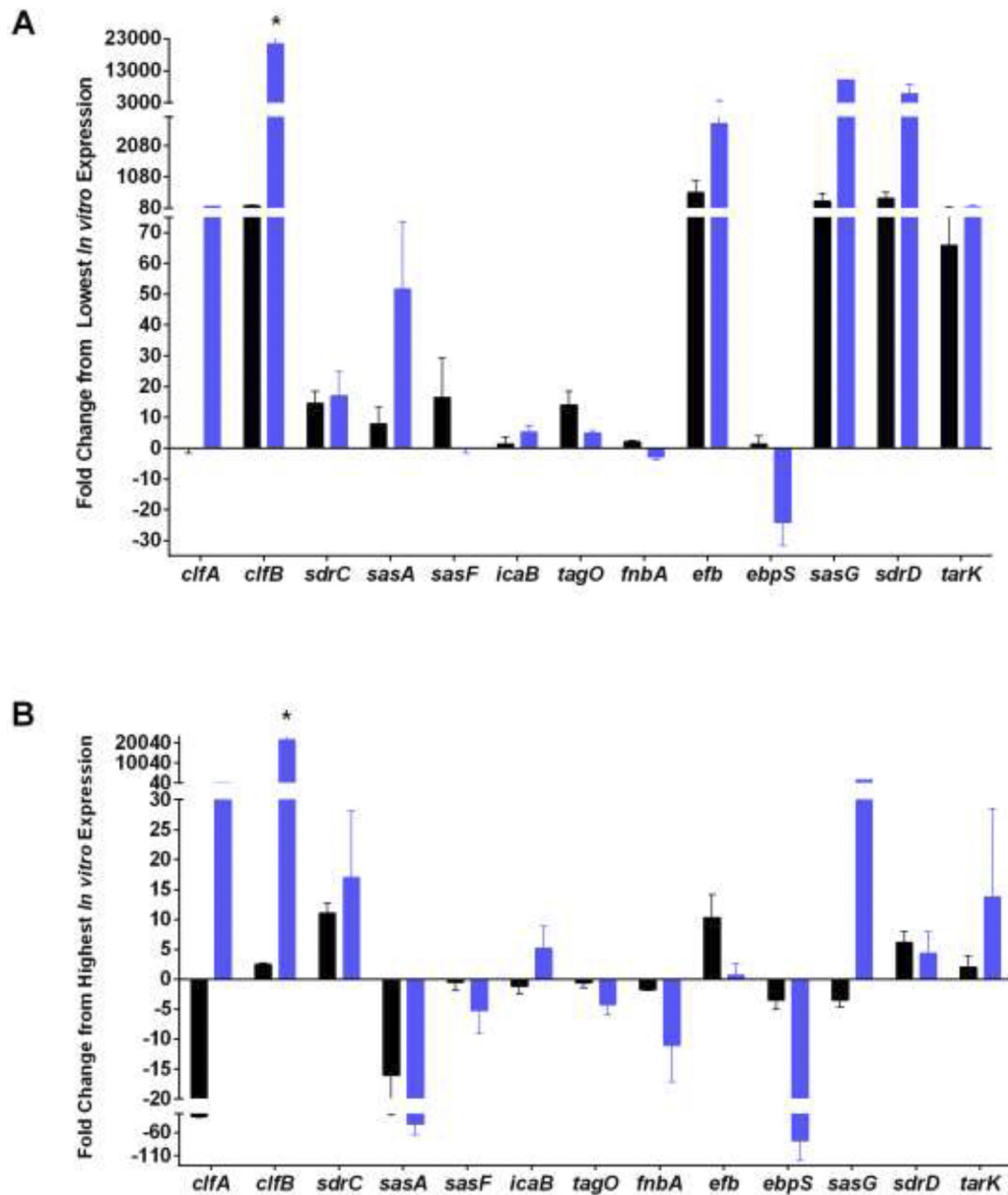


FIG 1 Protein and carbohydrate adhesin genes in a cotton rat nasal colonization model. Transcripts were assessed relative to 16S rRNA, and results from nasal colonization were compared to the minimum expression *in vitro* (A) and the maximum expression *in vitro* (B). *In vitro* growth conditions are described in Table S2 in the supplemental material. Analysis was performed for two *S. aureus* strains, SF8300 (black bars) and ARC633 (blue bars). These results are the means of three independent experiments with 12 animals/experiment. *, $P < 0.05$ (Student's *t* test). *clfB* expression was not detected *in vitro*, and so calculations for *clfB* expression were based on an *in vitro* threshold cycle C_T value equal to the limit of detection (40 cycles).

infected intranasally with isogenic mutants, and CFU were compared to exposure to wild type (WT) 4 days postinfection (Fig. 6). Only the $\Delta sdrC$ mutant showed a trend toward a reduction (~ 0.5 log) in nasal colonization, and this trend was not significant. When both *sdrC* and *clfB* (an adhesin shown to play a role in nasal colonization [21, 30]) were deleted ($\Delta sdrC \Delta clfB$), a significant reduction in CFU in the nares was observed.

Mutant strains were compared to WT for a change in heart CFU following i.v. infection. Knockout mutants for *clfA*, *sdrC*, *isdB*, and *hla* all resulted in decreased heart CFU, consistent with increased expression observed in thromboembolic lesions (Fig. 7). Knockout mutants of genes that were downregulated or un-

changed in thromboembolic lesions did not impact CFU recovery from the heart, with the exception of ΔspA (Fig. 7). It should be noted that all isogenic mutants were determined to have no growth defects in TSB compared to WT SF8300 (data not shown).

DISCUSSION

Understanding how *S. aureus* regulates gene expression as it transitions from colonization to infection is critical to understanding how *S. aureus* causes disease. We used qRT-PCR to examine transcript levels of known or putative virulence determinants in bacteria isolated from relevant animal models. *In vivo* bacterial gene expression data are typically presented relative to an arbitrary *in*

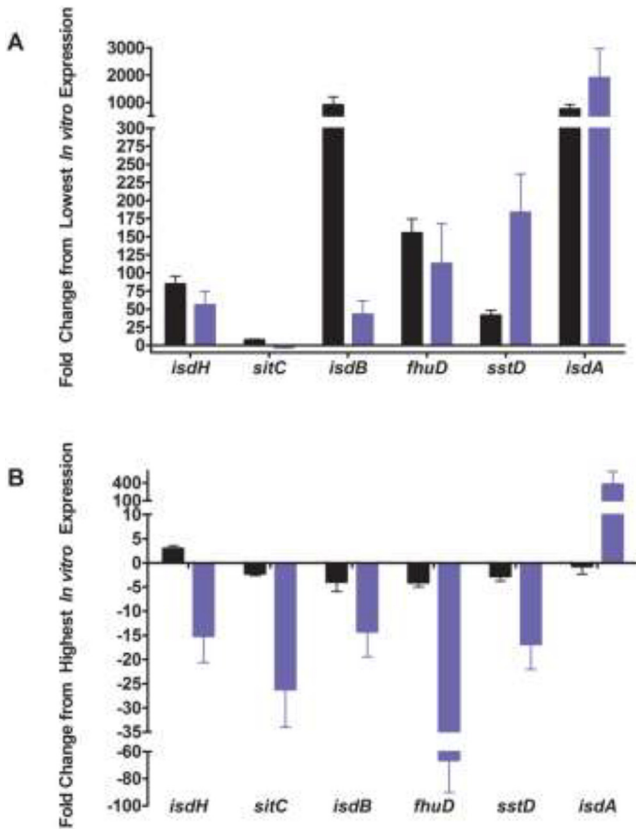


FIG 2 Metal cation acquisition gene expression in a cotton rat nasal colonization model. Transcripts were assessed relative to 16S rRNA, and results from nasal colonization were compared to the minimum expression level *in vitro* (A) and the maximum expression level *in vitro* (B). *In vitro* growth conditions are described in Table S2 in the supplemental material. Analysis was performed for two *S. aureus* strains, SF8300 (black bars) and ARC633 (blue bars). These results are the means of three independent experiments with 12 animals/experiment. *, $P < 0.05$ (Student's *t* test).

in vitro condition, making interpretation of gene expression data in disease states difficult. To avoid this limitation, gene expression patterns were compared directly from nasal colonization to bacteremia to thromboembolic lesions. This reduced the need to compare expression to an *in vitro* condition and allowed for the assessment of gene expression changes in the bacterium at differ-

ent stages of infection (e.g., commensal to pathogenic). Out of necessity, nasal colonization expression was compared to *in vitro* expression to establish a baseline for future comparisons.

Given the well-documented role of adhesins during nasal colonization (14–17, 21–23, 30, 31), it was not surprising that several adhesin genes were dramatically upregulated (5- to 21,000-fold) in at least one strain relative to their highest expression *in vitro*. For example, ClfB, SdrC, SdrD, and SasG have been proposed to play roles in nasal colonization, in either *in vivo* (21) or *in vitro* (22, 23) studies. A central role for SdrC and ClfB in nasal colonization was confirmed, as the $\Delta sdrC \Delta clfB$ double mutant showed a significant CFU reduction relative to wild-type SF8300. To our knowledge, this is the first direct evidence of a role for SdrC in nasal colonization *in vivo*. SdrC has been reported to bind β -neurexin, a protein found primarily in brain tissue, not on nasal epithelium (32), suggesting an alternate ligand for SdrC. In addition to *clfB* and *sdrC*, transcript levels of *clfA*, *icaB*, *sdrD*, *tarK*, *sasG*, and *efb* were increased, suggesting that they also play a role in nasal colonization. These results support the hypothesis that nasal colonization is a multifactorial process requiring numerous adhesins (23).

Nearly all metal cation acquisition genes analyzed during nasal colonization showed decreased expression relative to their highest *in vitro* expression, although expression was increased above their lowest *in vitro* level. This was not unexpected, as the nares are a low-iron environment (33), but their environment may not be as metal ion restricted as RPMI medium. Although *isdH* expression exhibited a slight increase (3-fold) during nasal colonization compared to the maximum *in vitro* expression, there was no change in bacterial CFU in the nares of cotton rats colonized with $\Delta isdH$ versus WT. This does not rule out a role for IsdH in iron acquisition, but it could reflect the redundant nature of *S. aureus* iron acquisition systems (33). Similar to published results, *hla* expression was low in the nares (14), and the Δhla mutant did not affect bacterial CFU in the nares, suggesting *hla* does not play a significant role in nasal colonization. However, due to the multifactorial nature of nasal colonization, a role for the toxins cannot be ruled out. Finally, the immune evasion proteins exhibited a large increase in expression in nasal colonization. This was expected, as this is the bacterium's first encounter with the immune system.

Several adhesin genes (*fmbA*, *sdrC*, *iCab*, *sasA*, *tagO*, *efb*, and *ebpS*), all of the immune evasion genes, and both toxins increased in expression at least 2-fold in the bloodstream of mice relative to

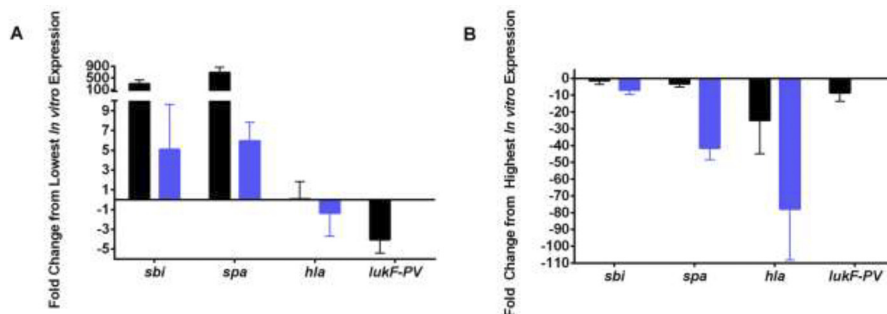


FIG 3 Immune evasion and exotoxin gene expression in a cotton rat nasal colonization model. Transcripts were assessed relative to 16S rRNA, and results from nasal colonization were compared to the minimum expression level *in vitro* (A) and the maximum expression level *in vitro* (B). *In vitro* growth conditions are described in Table S2 in the supplemental material. Analysis was performed for two *S. aureus* strains, SF8300 (black bars) and ARC633 (blue bars). These results are the means of three independent experiments with 12 animals/experiment. *, $P < 0.05$ (Student's *t* test).

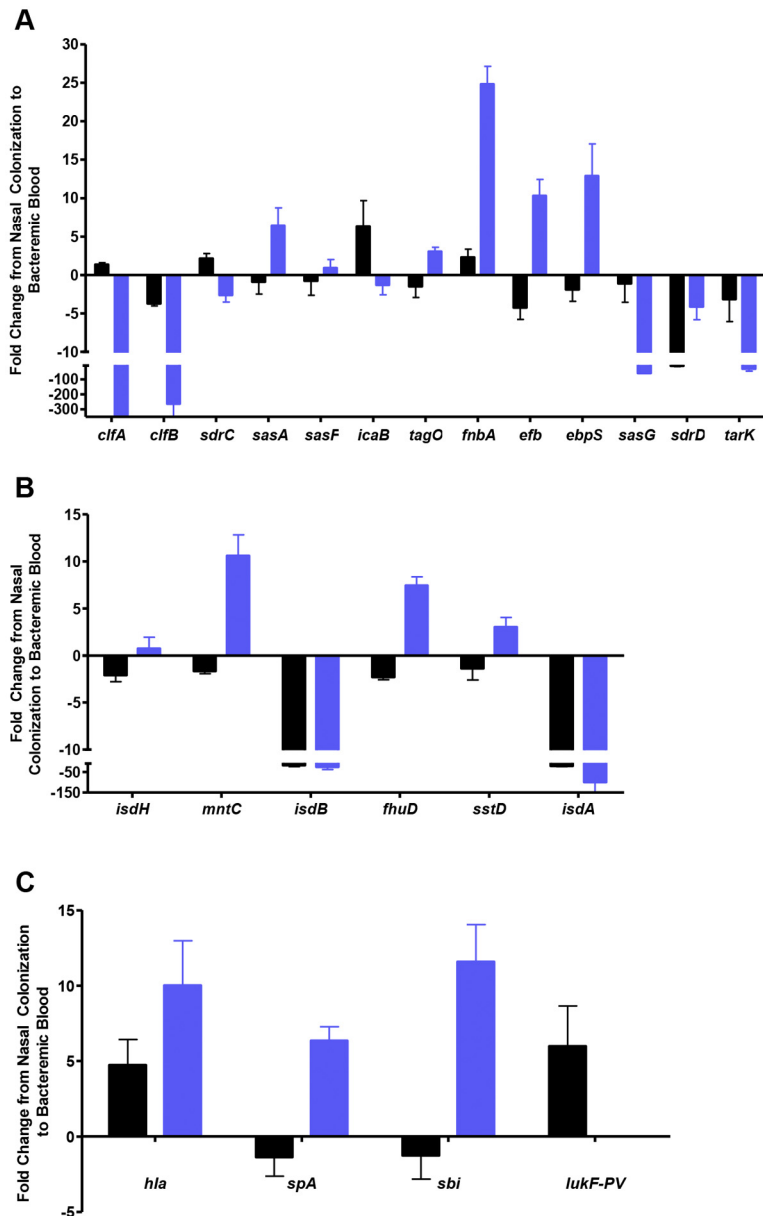


FIG 4 Differential gene expression between nasal colonization and early bacteremia. Transcripts were assessed relative to 16S rRNA, and results from the bloodstream were compared to the expression results from nasal colonization for protein and carbohydrate adhesins (A), metal cation acquisition genes (B), and immune evasion genes and exotoxins (C). Analysis was performed for two *S. aureus* strains, SF8300 (black bars) and ARC633 (blue bars). These results are the means of three independent experiments with 10 animals/experiment. *, $P < 0.05$ (Student's *t* test). *clfA*, *clfB*, and *sasG* expression was not detected in the blood samples, and so calculations for *clfA*, *clfB*, and *sasG* expression were based on the threshold cycle (C_T) value equal to the limit of detection (40 cycles).

nasal colonization, while the metal cation acquisition genes were largely unchanged or decreased relative to expression in the nares. Increased adhesin gene expression suggests that, upon exposure to the bloodstream, *S. aureus* begins to adhere to host cells or tissue. While a role for *icaB* during bacteremia has been reported (34), *sdrC*, *sasA*, *tagO*, *efb*, and *ebpS* have not been reported to play a role during bacteremia. Several of these adhesins have been studied in both *in vitro* and *in vivo* systems, and those results, combined with the results presented here, provide insights into a role for these genes in *S. aureus* pathogenesis. For example, FnBA and TagO have been reported to play a role in adherence to endothelial

cells (29, 35, 36). Although SasA has not been implicated in bloodstream infection, it has been reported that SasA antibodies are detected in convalescent patient sera, providing further evidence that SasA is expressed by *S. aureus* during systemic infection (37). These data suggest *S. aureus*, even early in bacteremia, rapidly expresses adhesins to facilitate binding to endothelial surfaces, presumably to escape the bloodstream and colonize host tissues. The three other upregulated adhesin genes, *efb*, *ebpS*, and *sdrC*, have not been implicated in *S. aureus* bacteremia; however, our data suggest a role for these genes in *S. aureus* bacteremia and/or endothelial adherence.

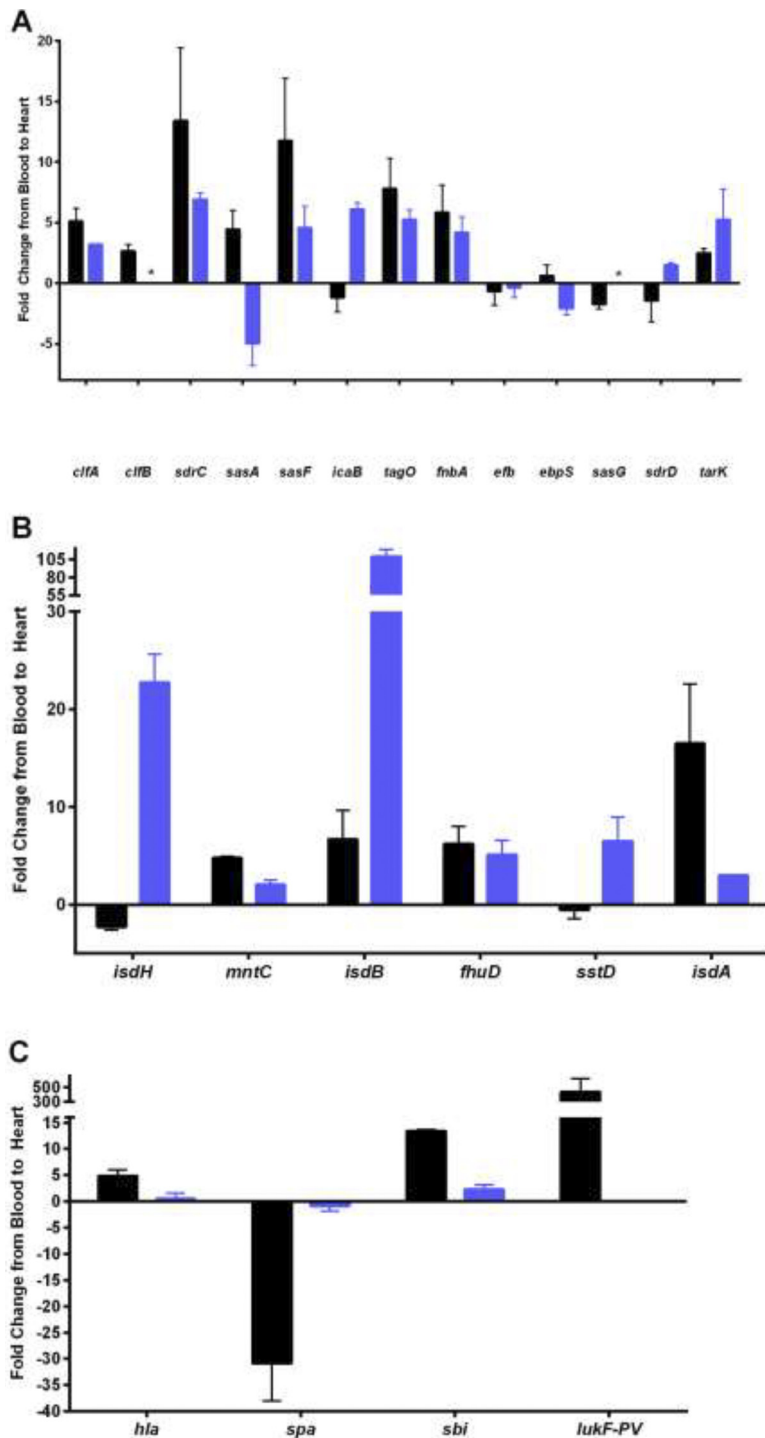


FIG 5 Differential gene expression in the transition from the bloodstream to heart tissue. Transcripts were assessed relative to the 16S rRNA, and results from the heart tissue were compared to the expression results from the bloodstream for protein and carbohydrate adhesins (A), metal cation acquisition genes (B), and immune evasion genes and exotoxins (C). Analysis was performed for two *S. aureus* strains, SF8300 (black bars) and ARC633 (blue bars). These results are the means of three independent experiments with 10 animals/experiment. *, $P < 0.05$ (Student's *t* test). *clfB* and *sasG* expression was not detected in the blood samples or the heart samples; therefore, expression numbers are not reported for these genes.

The metal cation acquisition proteins (except for *isdA* and *isdB*) were still highly expressed in the bloodstream, where they aid in scavenging iron. The difference in *isdB* and *isdA* expression relative to nasal colonization could highlight differences in nutri-

ent availability and host iron-scavenging proteins in the nares versus the blood (38). Increased expression of *sbi*, *spa*, and *lukF-PV* suggests that the bacterium is actively engaged in evading the host immune system upon introduction into the bloodstream. In-

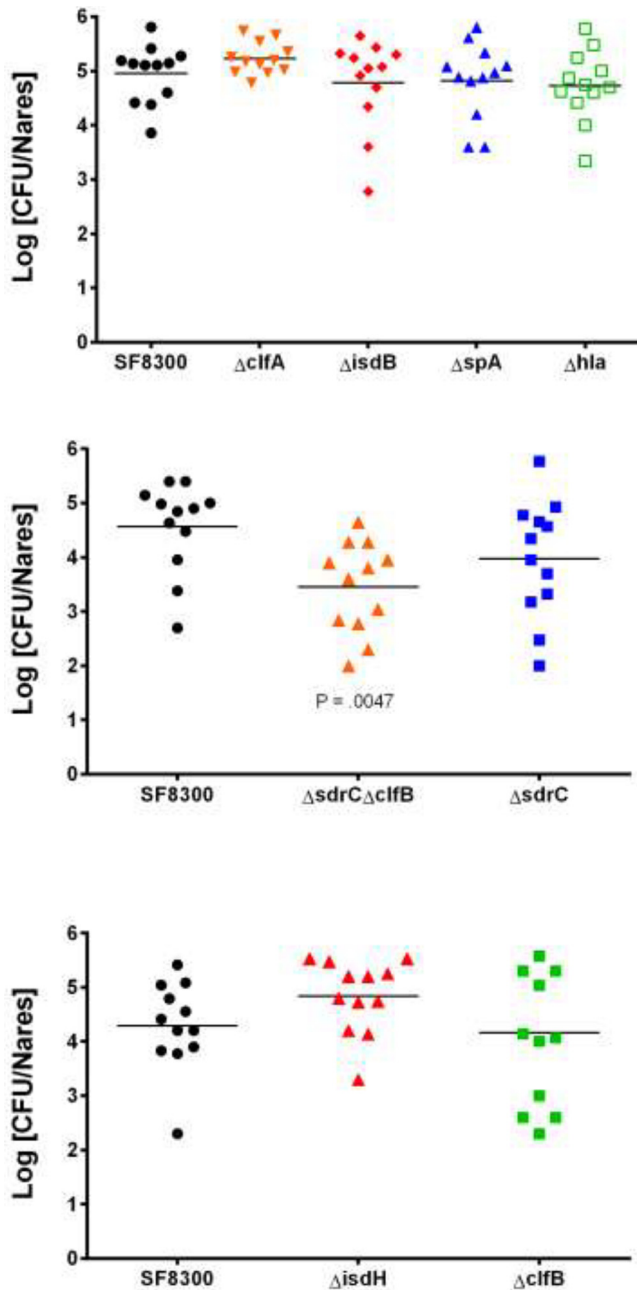


FIG 6 CFU enumeration of SF8300 wild-type and mutant strains in a cotton rat nasal colonization model. Groups of 12 cotton rats were challenged intranasally with 5×10^5 CFU *S. aureus* in $10 \mu\text{l}$ ($5 \mu\text{l}$ per nostril). For $\Delta sdrC \Delta clfB$, $P = 0.0047$ (Mann-Whitney test). Data shown are from a single replicate of three independent experiments.

creased *hla* expression during bacteremia supports previous studies describing a role for alpha-toxin (AT) in bloodstream infections (39).

Transition from bacteremia to heart tissue led to drastically increased expression of most adhesin genes and all metal cation acquisition, toxin, and immune evasion genes, with the exception of *spa*, relative to bloodstream expression. Increased adhesin gene expression may be expected, as these proteins are generally thought to facilitate adherence to and colonization of host tissues

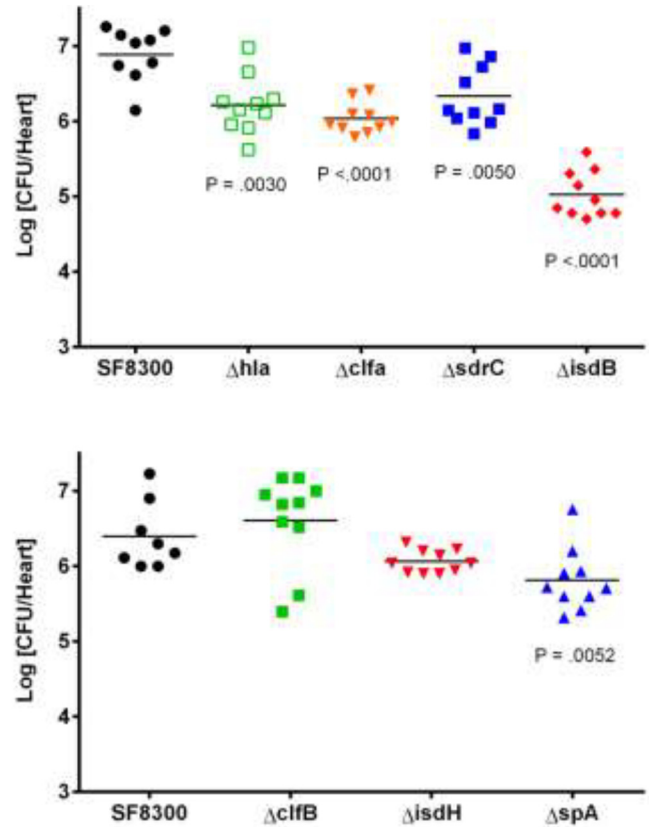


FIG 7 CFU enumeration of SF8300 wild-type and mutant strains in a murine thromboembolic lesion model. Groups of 10 female BALB/c mice were challenged intravenously (i.v.) with $200 \mu\text{l}$ of *S. aureus* (5×10^7 CFU) by tail vein injection. P values were as follows: Δhla , 0.0030; $\Delta clfA$, <0.0001 ; $\Delta sdrC$, 0.0050; $\Delta isdB$, <0.0001 (Mann-Whitney test). Data shown are from a single replicate of three independent experiments.

(40, 41). Although some of the upregulated adhesins have been shown to play a role in establishing organ lesions (ClfA [26, 27], FnbA [35], and TagO [29]), many of them do not have a reported role in organ lesion formation. Increased expression of *sdrC* (13-fold), *sasA* (4-fold), and *sasF* (11-fold) are of particular interest in that little is known about their roles in *S. aureus* virulence. While a $\Delta sdrC$ mutant strain led to a relative decrease in CFU recovered from heart lesions compared to WT, further experiments are required to elucidate the role of these putative adhesins in severe *S. aureus* infections. The upregulation of all metal ion acquisition genes (with the exception of *isdH* and *sstD* in strain SF8300) indicates that heart lesions may be more iron limiting than blood. Previous studies have shown iron content varies among organs, with heart muscle containing less iron than other organs, although free blood iron levels were not determined in those studies (42). The expression of *spa* in the heart is decreased relative to that in the bloodstream; however, Δspa resulted in decreased heart CFU relative to infection with WT. This may be due to high *spa* expression in the bloodstream; therefore, a decrease in *spa* between the bloodstream and the heart still results in a significant amount of protein A present on the bacterial surface. Alternatively, perhaps Δspa reduces bacterial survival in the blood, resulting in lower bacterial numbers invading the heart tissue. Although AT has been shown to be important in establishing *S. aureus* infections,

including infective endocarditis (43), there is no evidence that it plays a role in thromboembolic lesions. The data presented here suggest AT plays a role in infecting the heart tissue, both through increased transcript levels and decreased CFU recovered from heart tissue following infection with Δhla relative to infection with WT. One hypothesis for AT's role in establishing the heart lesions involves its activation of ADAM10-mediated E-cadherin cleavage, resulting in vascular leakage and bacterial invasion of heart tissue (44).

To our knowledge, these results are the first example of a study designed to directly compare virulence factor expression by a bacterium during commensal and pathogenic states. Additionally, this study has shown that this type of analysis is possible, and valuable information can be garnered by comparing expression patterns between colonization and disease states, laying the groundwork for large-scale microarray studies for a broader view of *S. aureus* as a commensal and a pathogen. Recently, a microarray study examining *S. aureus* isolated from human skin infections was performed and, as expected, many of the upregulated genes correlated well with our *in vivo* results (e.g., *hla*, *isdB*, *isdA*, and *sitC*) (13). The results obtained in this study support previous reports describing the involvement of individual *S. aureus* genes and proteins in the three disease states, and they provide new information about genes which may play a role in nasal colonization (*icaB*, *clfA*, and *efb*), early bloodstream infection (*sdrC*, *sasA*, *tagO*, *fnbA*, *efb*, *ebpS*, *mntC*, *fhuD*, and *sstD*), and thromboembolic lesions (*sdrC*, *sasA*, *sasF*, *isdH*, *mntC*, *sstD*, *fhuD*, *spA*, *sbi*, and *hla*). The steadily increasing expression of *sdrC*, *fnbA*, *fhuD*, *sstD*, and *hla* (in at least one strain) from nasal colonization to bacteremia to heart lesions suggests a role for these genes in the transition from commensal to pathogen. The expression patterns of the chosen genes often varied between the strains tested, highlighting *S. aureus* diversity and stressing the need to evaluate multiple clinical isolates when identifying important virulence factors. The information collected provides a basic understanding of *S. aureus* disease pathogenesis and how a commensal becomes a pathogen, and this understanding may ultimately lead to the development of targeted therapeutics.

MATERIALS AND METHODS

***S. aureus* strains, media, and growth.** SF830 is a prototypical USA300-0114 community-associated methicillin-resistant *S. aureus* strain (45). ARC633 (CC15; MSSA) is a nasal colonization isolate provided by David A. Bruckner, UCLA Medical Center. Bacteria were cultured with shaking (250 rpm) at 37°C in TSB for growth curves and for *in vitro* gene expression cultures. For RNA purification from *in vitro* cultures, samples were mixed 1:2 with RNeasy Protect (Qiagen), incubated at room temperature for 5 min, and pelleted by centrifugation. The pellet was resuspended in 1 ml of TRIzol (Invitrogen). The TRIzol-treated samples were lysed by using a FastPrep 24 homogenizer with lysing matrix B tubes (MP Biomedicals). RNA purification was performed as described below. Animal challenge stocks were prepared by growing bacteria overnight in TSB, then diluting cultures into fresh TSB and growing until the optical density at 600 nm reached ~0.8. The bacteria were collected by centrifugation and resuspended in phosphate-buffered saline (PBS; Invitrogen) plus 10% glycerol and frozen at -80°C in aliquots for use in all animal studies.

Cotton rat nasal colonization model. Cotton rats ($n = 12$) were challenged intranasally with 5×10^5 CFU *S. aureus* in 10 μ l (5 μ l per nostril). Four days postchallenge, the nares were harvested and placed into 20 ml of RNeasy Protect (for RT-PCR) and incubated for 5 min, or placed into 2 ml of PBS plus 0.1% Tween 20 (for CFU enumeration). For RT-PCR, nares were placed into 10 ml of TRIzol (Invitrogen) and vigorously vortexed to

remove bacteria. The TRIzol-treated samples were lysed using the FastPrep 24 homogenizer with lysing matrix B tubes (MP Biomedicals). RNA purification was performed as described below. All RT-PCR data are reported as means of 3 independent experiments using 12 animals/experiment. For CFU enumeration, nares were homogenized using a Polytron PT-10-35 GT apparatus. The samples were serially diluted and plated onto Staph Chrome agar plates (BD Biosciences).

All animal use protocols were reviewed and approved by MedImmune's IACUC and complied with the animal welfare standards of the USDA, Guide for the Care and Use of Laboratory Animals, and AAALAC International.

Murine bacteremia. Female BALB/c mice ($n = 10$) were challenged by tail vein injection of 1×10^8 CFU of *S. aureus* (200 μ l). One hour post-challenge, blood was collected from and pooled in 20 ml of RNeasy Protect. The bacteria were pelleted by centrifugation, resuspended in 1 ml of TRIzol, and treated as described above. All RT-PCR data are reported as means of 3 independent experiments using 10 animals/experiment.

Murine sepsis. Female BALB/c mice ($n = 10$) were challenged by tail vein injection of 1×10^7 CFU *S. aureus* (200 μ l). Fourteen hours post-challenge, hearts were harvested and placed into 20 ml of RNeasy Protect (for RT-PCR) and incubated for 5 min, or tissue was placed in 1 ml of PBS plus 0.1% Tween 20 (for CFU enumeration). Bacteria and heart tissue were lysed, and RNA was purified as described above. All RT-PCR data are reported as means of 3 independent experiments using 10 animals/experiment. For CFU enumerations, hearts were homogenized in 1 ml of PBS plus 0.1% Tween 20 in lysing matrix A tubes (MP Biomedicals) in a FastPrep 24 homogenizer. Samples were serially diluted and plated onto trypticase soy agar plates (BD Biosciences).

Construction of in-frame gene deletions. In-frame deletions of selected genes were constructed by allelic replacement using pKOR1 (46). Primers X1-X2 (see Table S3 in the supplemental material) were used to amplify approximately 1,000 bp that corresponded to the first 84 to 153 nucleotides from the start codon and flanking 5' region. Primers X3-X4 were used to amplify approximately 1,000 bp that corresponded to the last 33 to 159 nucleotides from the stop codon and flanking 3' region (see Table S3). X1-X2 and X3-X4 PCR products were spliced together by overlap PCR using primers X5 and X6. Attachment sites (attB), appended to 5' ends of primers X5 and X6 were recombined with the attP sequences flanking a lambda recombination cassette on pKOR1 in the presence of bacteriophage lambda integrase and *Escherichia coli* integration host factor (Clonase; Invitrogen) and electroporated into *E. coli*. The in-frame deletion constructs were electroporated into *S. aureus* RN4220 and then transduced with $\Phi 11$ into SF8300. Allelic replacement was performed as described elsewhere (46). Allelic replacement mutants were identified by PCR and DNA sequencing using primers X1 to X4 and primers X1, X4, S1, and S2, respectively (see Table S3). Multiple gene deletions were carried out sequentially.

RNA preparation. Chloroform (200 μ l) was added to 1 ml of lysed bacteria and centrifuged at $14,000 \times g$, and the top clear layer was removed, added to 1 ml of isopropanol, and then incubated at -20°C overnight. RNA was pelleted by centrifugation ($14,000 \times g$) for 30 min. The pellet was washed with 70% ethanol, dried, resuspended in 100 μ l of RNase-free water, and digested with RNase-free DNase (Promega) for 1 h. Following digestion, 1 ml of TRIzol and 200 μ l of chloroform were added. RNA precipitation and DNase digestion procedures were repeated. RNA was then purified using the RNeasy minikit (Qiagen).

RT-PCR. RNA was reverse transcribed into cDNA by using the SuperScript III cDNA synthesis kit (Invitrogen). TaqMan primers (see Table S4 in the supplemental material) containing a 6-carboxyfluorescein reporter and nonfluorescent quencher were designed using the TaqMan design tool (Life Technologies). cDNA samples were assayed in triplicate using 16S rRNA as a control. Samples were assayed using TaqMan universal PCR master mix (Applied Biosystems) on an Applied Biosystems 7900HT apparatus with standard cycling protocols and analyzed using SDS software. Relative expression values were calculated using the $\Delta\Delta C_T$ method

with 16S RNA as the normalizer (47–49). 16S RNA was determined to be the most stable housekeeping gene in our experiments, based on expression under a variety of *in vitro* growth conditions (rich medium, minimal medium, or in the presence of serum).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02272-14/-/DCSupplemental>.

- Figure S1, DOCX file, 0.02 MB.
- Figure S2, DOCX file, 0.1 MB.
- Table S1, DOC file, 0.1 MB.
- Table S2, DOC file, 0.05 MB.
- Table S3, DOCX file, 0.01 MB.
- Table S4, DOCX file, 0.01 MB.

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