MgrA Activates Expression of Capsule Genes, but Not the α-Toxin Gene in Experimental *Staphylococcus aureus* Endocarditis

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Background. *Staphylococcus aureus* produces numerous virulence factors but little is known about their in vivo regulation during an infection.

Methods. The production of capsule and α-toxin, and the expression of their respective genes, *cap5* and *hla*, were analyzed by comparing CYL11481 (derivative of Newman) and its isogenic regulatory mutants in vitro. The temporal expression of *cap5* and *hla* and the regulatory genes in vivo was carried out using a rat infective endocarditis model.

Results. In vitro analyses showed that capsule was positively regulated by MgrA, Agr, Sae, ArlR, and ClpC, and negatively by CodY and SbcDC. The α-toxin was positively regulated by MgrA, Agr, Sae, ArlR, and SbcDC but negatively by ClpC and CodY. In vivo analyses showed that *cap5* expression correlated best with *mgrA* expression, whereas *hla* expression correlated best with *sae* expression. Mutation in *mgrA* drastically reduced *cap5* expression in vivo.

Conclusions. Our results suggest that, in vitro, Agr is the most important regulator for capsule and α-toxin production, as well as for *cap5* transcription, but SaeR is the most critical for *hla* transcription. However, in vivo, MgrA is the major transcriptional regulator of capsule, but not α-toxin, whereas *saeR* expression correlates best with *hla* expression.

Keywords. *Staphylococcus aureus*; virulence; infective endocarditis model; mgrA; capsule; α-toxin.

Staphylococcus aureus causes many diseases, ranging from superficial skin infections to severe and life-threatening infections such as endocarditis, septic arthritis, and sepsis [1]. Endovascular infections caused by *S. aureus* are often associated with implanted medical devices, such as prosthetic heart valves and vascular catheters [2]. The pathogenicity of *S. aureus* is a very complex process involving coordinated expression of numerous secreted enzymes, toxins, antiphagocytic capsular polysaccharides (CPs) and adhesins [1].

These virulence factors are coordinately regulated by a network of regulatory genes, which include 2-component systems (TCSs) and various transcriptional regulators [3, 4]. The accessory gene regulator (*agr*) and staphylococcal accessory regulator (*sarA*) represent the most well-characterized global regulatory elements in *S. aureus*, which coordinate synthesis of multiple virulence factors [3, 5]. MgrA is a multiple gene regulator belonging to the SarA family, which has been shown to directly regulate several virulence genes by binding to their respective promoter regions [6–8]. Several virulence factors have been shown to be upregulated by MgrA, such as capsular polysaccharides, sortase A, serine proteases, leukotoxins, and α-toxin. In contrast, several cell wall–associated or surface-associated proteins are downregulated by MgrA [9, 10]. MgrA has also been shown to regulate genes involved in bacterial metabolism, antibiotic resistance, and autolysis [7–9]. Regulation by MgrA is affected by the redox and phosphorylation state of the regulator [11, 12].

MgrA is critical for *S. aureus* pathogenesis, as shown in a murine abscess model and a murine sepsis arthritis model [11, 13].

Capsule is one of several virulence factors that contribute to *S. aureus* pathogenicity, which has been shown to attenuate virulence in experimental infective endocarditis (IE) [14, 15]. This cell surface molecule is produced by most *S. aureus* strains, with type 5 (CP5) or type 8 (CP8) being the dominant serotypes. The *cap5* and *cap8* operons involved in the biosynthesis of CP5 and CP8, respectively, are allelic, and each contains 16 genes in which 4 serotype-specific genes are flanked by common genes [16]. The nearly identical promoter regions of the *cap5* and *cap8* operons indicate that their mechanisms of regulation are similar. Staphylococcal capsule has been shown to be affected by various environmental conditions such as carbon dioxide, iron limitation, pH, oxygen tension, and in vivo environmental cues [14, 15]. We have previously identified several genes that regulate capsule expression in vitro. Among these, Agr, MgrA, and ArlR are activators, whereas ClpC, Sae, SbcDC, and CodY are repressors of capsule production [6, 9, 17–22]. ArlR is the response regulator of the ArlRS TCS that activates the expression of capsule primarily through an MgrA-dependent pathway in vitro in strain Newman [16]. SbcDC has been shown to be involved in DNA repair in *Escherichia coli* [23]; however, in *S. aureus,* SbcDC represses capsule by negatively regulating the *arl* locus [19]. CodY, found in *Bacillus subtilis* and many low G + C gram-positive bacteria, is a pleiotropic regulator sensing nutrient limitation [24] that represses capsule in *S. aureus* [18, 20]. Recently, we reported that ClpC divergently regulated capsule via *sae*-dependent repression or via *codY*-dependent activation [21].

Staphylococcus aureus α-toxin, encoded by the *hla* gene, is a pore-forming extracellular protein that targets a variety of host cell types, and is tightly regulated by multiple genes in *S. aureus* [25]. The regulation of *hla* has been extensively studied in vitro. These studies suggest that *agr* exerts a direct positive impact on *hla* expression, whereas SarA positively affects *hla* expression indirectly by both *agr*-dependent and *agr*-independent pathways [26–28]. The TCS encoded by *saeS* and *saeR* positively regulates the expression of *hla* at the transcriptional level [29, 30]. MgrA has been shown to activate *hla* by direct promoter binding or through activation of *agr* [10].

Virulence gene regulation in *S. aureus* has mainly been dissected only under in vitro conditions, and little is known regarding gene regulation under in vivo conditions. An in vivo study on *hla* gene regulation in endovascular infection by Xiong et al [31] suggests that *hla* expression is more dependent on Sae than on Agr and/or SarA. The results of a transcriptional analysis using a soft-tissue guinea pig infection model by Goerke et al [27] also supports the notion that Sae is the key regulator of *hla* expression in strain Newman and RN6390. Using a rabbit IE model, van Wamel et al [32] showed that Agr, but not SarA, significantly affected capsule gene transcription

in vivo. However, these in vivo studies focused on the effect of 1 or 2 regulatory genes at 1 fixed time-point during infection. To better understand virulence gene regulation, we investigated the effect of a large cadre of regulators known to affect capsule in vitro on *cap5* gene expression in a rat IE model at 3 distinct time-points during disease development. We found that, despite its strong effect in vitro, Agr was not a key regulator of *cap5* gene expression in vivo; instead, MgrA was pivotal for *cap* gene transcription in vivo. We also analyzed *hla* gene expression in our study.

MATERIALS AND METHODS

Bacterial Strain and Plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. The *S. aureus* strain CYL11481, in which the proline at the 18th codon of *saeS* in strain Newman—that is, Newman *saeS*(P18L)—has been replaced with leucine, was used as the parental strain (21). Transduction between *S. aureus* strains was carried out by phage 52A or 80α. The isogenic mutants of CYL11481 were constructed by phage transduction from their respective parents (Table 1). All the mutants were verified by polymerase chain reaction (PCR) amplification, and complemented strains were verified by plasmid digestion.

Rat IE Model

All experiments involving animals followed protocols that have been reviewed and approved by the institutional animal care and use committee at the University of Arkansas for Medical Sciences. Rats were purchased from Harlan Inc, and were housed in Institutional Animal Care and Use Committee–accredited animal care facilities at the University of Arkansas for Medical Sciences. The surgical procedures were carried out

Table 1. Strains and Plasmids Used in the Study

Strain or Plasmid	Description	Reference
Staphylococcus aureus		
Newman	Wild-type CP5 strain	T. Foster
CYL11481	Newman *saeS*(P18L)	[21]
CYL12659	CYL11481 Δ*mgrA*	This study
CYL12676	CYL11481 Δ*mgrA* (pCL52.2)	This study
CYL12678	CYL11481 Δ*mgrA* (pTL2989)	This study
CYL12661	CYL11481 Δ*agr*	This study
CYL12752	CYL11481 Δ*arlR*	This study
CYL12723	CYL11481 Δ*codY*	This study
CYL11489	CYL11481 Δ*clpC*	This study
CYL12750	CYL11481 Δ*sbcDC*	This study
CYL12367	Newman Δ*saePQRS*	[22]
Plasmids		
pCL 52.2	Cloning vector	[6]
pTL2989	pCL 52.2 with *mgrA*	[6]

under full anesthesia, as well as local delivery of lidocaine. Rats were euthanized by exposure to 100% carbon dioxide in a closed chamber.

A well-characterized experimental rat IE model was used in the present study [33]. Sprague-Dawley female rats (220–240 g each) were anesthetized with an isoflurane-oxygen gas mixture (1:1 ratio) during the surgery. In brief, an indwelling polyethylene catheter was positioned in the left ventricle of each animal via the retrograde transcarotid artery approach, with the tip passing across the aortic valve, to induce sterile vegetations. At 48 hours after catheterization, animals were infected iv through the tail vein with an inoculum of 1×10^4 colony-forming units (CFU)/rat of strain CYL11481 or its isogenic *mgrA* mutant (this inoculum was chosen after extensive pilot studies identified an appropriate ID_{90}). Animals were sacrificed at days 1, 2, and 3 after infection. Vegetations were collected immediately in sterile tubes and frozen in dry ice until RNA isolation (see below). Only samples from rats with proper catheter placement and macroscopic vegetations were used for the study. Groups of 3 rats per time-point were used for the study, and each experiment was repeated in duplicate or triplicate.

RNA Isolation and Quantitative Reverse Transcription PCR

For in vivo transcriptional analysis, vegetations in each tube were suspended in 1 mL of sterile phosphate-buffered saline (pH 7.4) and homogenized with a mortar and pestle, and serial dilutions of 100 μL were plated on tryptic soy agar plates and incubated at 37°C for 24 hours for quantitative cultures (expressed as mean $\log_{10}$ CFU/g tissue ± SD). The remaining sample was processed for RNA isolation as described previously [34] using RNAzol-RT (Molecular Research Center, Inc) with some modifications. After homogenization, tissues were pelleted at 500g for 5 minutes, and supernatants containing bacterial cells were mixed with an equal volume of an ice-cold 1:1 mixture of ethanol-acetone and kept at −20°C for 2 hours or overnight. Cells were pelleted, washed 2 times with TNE buffer (50 mM Tris [pH 7.6], 150 mM NaCl, and 5 mM EDTA) and suspended in 50 μL of TNE buffer with 2.5 M NaCl. Cells were digested with 0.8 μg/μL of lysostaphin at 37°C for 5 minutes and immediately lysed with 1 mL RNAzol-RT, and RNA was isolated according to the manufacturer's instructions. Total RNA was further purified from the host tissue RNA using the MICROBEnrich Kit (Ambion AM1901). Purified bacterial RNA was checked for DNA contamination by running a no–reverse transcription (RT) PCR using *hu* gene primers for 25 cycles in a thermocycler. RNA was stored in −80°C until used. For day 1 sample, vegetations from 2 to 3 animals were pooled to obtain adequate quantity of RNA for analysis. For in vitro expression analysis of the *cap5* and *hla* genes, RNA was isolated as described previously [34] using RNAzol-RT from 1.5 mL overnight cultures adjusted to an OD_{660} of 2.0. The quantitative RT-PCR (qRT-PCR) was performed as described previously [9] using the primers listed in Supplementary Table 1.

In Vitro Capsule and α-Toxin Production Assays

Capsule quantification was done as described previously [6]. The semiquantitative assay of α-toxin was performed by spotting the filtered (0.22 μm) supernatants of overnight cultures onto 5% sheep blood agar plates (Remel, Lenexa), with the zones of clearance measured with calipers.

Statistical Analysis

Relative expression and bacterial burden data were analyzed by the GraphPad Prism version 4.00 using a Student t test ($P < .05$ considered as significant). Correlation coefficient (r^2) among the genes was analyzed by linear regression with best fit model in GraphPad Prism software.

RESULTS

Regulation of Capsule and α-Toxin In Vitro

Strain Newman has often been used in virulence regulation studies. However, genome sequencing revealed a single nucleotide variation in the *saeS* gene that resulted in unusually high expression of *sae*. Restoration of this point mutation resulted in strain CYL11481, ie, Newman *saeS*(P18L) [21]. Because Sae has been shown to affect many virulence regulators, we first assessed the effect of regulatory gene mutations in the *saeS*-corrected strain CYL11481 background on capsule and α-toxin production at the transcriptional as well as posttranscriptional levels. Capsule expression in CYL11481 was positively regulated (in decreasing order of relative impact) by Agr, ArlR, MgrA, ClpC, Sae, and negatively regulated by CodY and SbcDC (Figure 1*A* and 1*B*). For α-toxin, we found that (in decreasing order of effect) Sae, ArlR, Agr, MgrA, and SbcDC were positive regulators, and CodY and ClpC were repressors at the transcriptional level. Interestingly, the *agr* mutation has a strongest effect on α-toxin activity, suggesting that Agr is a key regulator of α-toxin at both the transcriptional and the posttranscriptional levels (Figure 2*A* and 2*B*).

Regulation of Capsule and α-Toxin In Vivo

To study gene regulation in vivo, we chose to focus on capsule because regulation of capsule has been studied extensively in vitro. A total of 7 regulatory genes, as well as the *cap5* and *hla* genes, were analyzed for their expression longitudinally during the temporal progression of experimental IE. The inclusion of the *hla* gene served as an appropriate comparison, as regulation of α-toxin has also been well studied in vitro, and regulation of *hla* in vivo has already been reported [27, 31]. As shown in Figure 3, we found in the parental strain that most of the genes of interest were strongly induced in vivo. The exceptions were
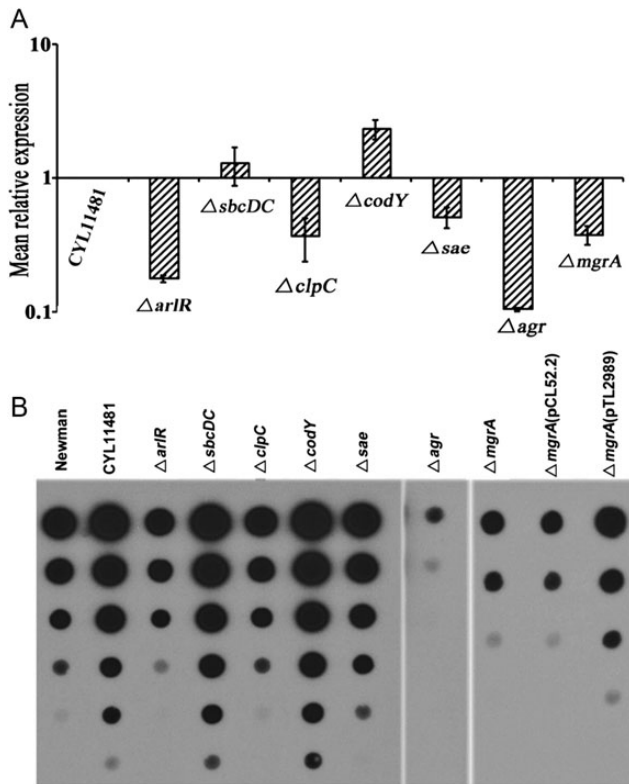
Figure 1. In vitro analysis of *cap*5 gene expression and capsule production. *A*, The expression of *cap5D* gene was analyzed by quantitative reverse transcription polymerase chain reaction for 18-hour cultures of CYL11481 (parent strain) and its isogenic mutants of *arlR* (CYL12752), *sbcDC* (CYL12750), *clpC* (CYL11489), *codY* (CYL12723), *sae* (CYL12367), *agr* (CYL12661), and *mgrA* (CYL12659). Expression levels are expressed relative to that of CYL11481, which was arbitrarily set at 1. Data represent the mean relative expression of duplicate experiments with standard deviation. *B*, Capsule production was assayed for 18-hour cultures of the strains listed above; strain Newman, and the complemented *mgrA* mutant. The crude capsule preparations were serially diluted 3-fold and assayed by immunoblotting with anti–type 5 capsule antibody.

the *clpC* and *codY* genes, which were both reduced, especially the *clpC* gene. Interestingly, RNAIII, the effector of *agr*, was reduced at days 1 and 3 but was highly increased at day 2. The expression of *cap5* and *mgrA* were at a relatively constant level over time, whereas *hla* and *saeR* were more variable, peaking at day 2. Overall, the expression of *cap5* correlated best with that of *mgrA* ($r^2 = 0.99$) and poorly with other regulators ($r^2 = 0.007–0.82$). The expression of *hla* matched best with that of *saeR* ($r^2 = 0.88$). These results suggest that MgrA is the major *cap5* regulator in vivo in this model, and that Sae is a key regulator of *hla* under these in vivo conditions.

Role of MgrA in *cap5* and *hla* Expression In Vivo
To further study the role of MgrA in the regulation of capsule and α-toxin in vivo, we constructed an *mgrA* mutant of strain CYL11481 and performed qRT-PCR to measure the expression
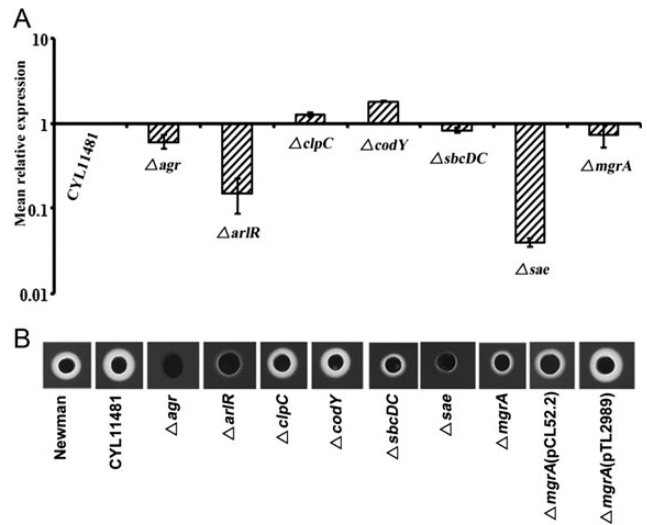

Figure 2. In vitro analysis of *hla* expression and α-toxin activity. *A*, The expression of the *hla* gene was analyzed by quantitative reverse transcription polymerase chain reaction as described in the legend of Figure 1*A* with the same set of strains. Data represent the mean relative expression of duplicate experiments with standard deviation. *B*, α-Toxin activity was evaluated by spotting 15 μL of filtered culture supernatants of the strains listed in Figure 1*B* on 5% sheep blood agar plates followed by incubation for 24 hours at 37°C. Zones of clearance on the blood agar plate indicate Hla activity.

profile of the *cap5* and *hla* genes during the progression of disease in the rat IE model. The mutant was verified by complementation to ensure that no second site mutation occurred during strain construction (Figure 1*B* and 2*B*). We also included RNAIII expression in the experiments, as MgrA has been shown to affect *agr* in vitro [10]. Our results showed that *cap5* gene expression in the *mgrA* mutant was highly reduced during the 3 days of infection suggesting that MgrA is a major regulator of capsule in vivo. On the other hand, the expression of *hla*
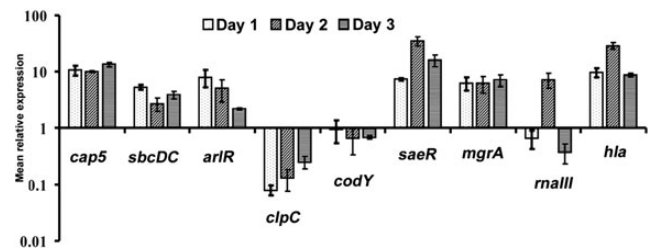

Figure 3. Gene expression in CYL11481 during infective endocarditis infection. RNA was isolated from groups of 3 rats infected with CYL11481 at days 1, 2, and 3 after infection, and each experiment was repeated 2–3 times. The expression of the genes indicated in the figure was assayed by quantitative reverse transcription polymerase chain reaction as described in the legend of Figure 1*A*.

Figure 4. Gene expression in *mgr*A mutant during infective endocarditis infection. RNA was isolated from groups of 3 rats infected with CYL12659 at days 1, 2, and 3 after infection, and each experiment was repeated 2 times. The expression of the genes indicated in the figure was assayed by quantitative reverse transcription polymerase chain reaction as described in the legend of Figure 1*A*.
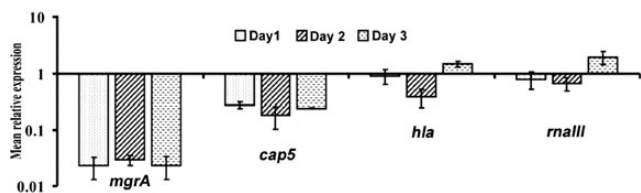
and RNAIII was reduced at day 2 but slightly increased in day 3. Comparing these results to those of the wild type shown in Figure 3, we concluded that the *mgr*A mutation had a negative effect on both *agr* and *hla* at day 2, but its effect at day 3 was unclear. Based on the expression profiles, we suggest that MgrA may have only a marginal and possibly an indirect effect on the expression of *agr* and *hla* in the rat IE model (Figure 4).

Mutation in *mgrA* Attenuated *S. aureus* Virulence in Experimental IE

The role of MgrA in virulence in endocarditis has not been studied. Here, we quantified the temporal bacterial burden within cardiac vegetations in animals with IE infected with either the parent strain or its isogenic *mgr*A mutant strain. As shown in Figure 5, animals infected with the wild-type
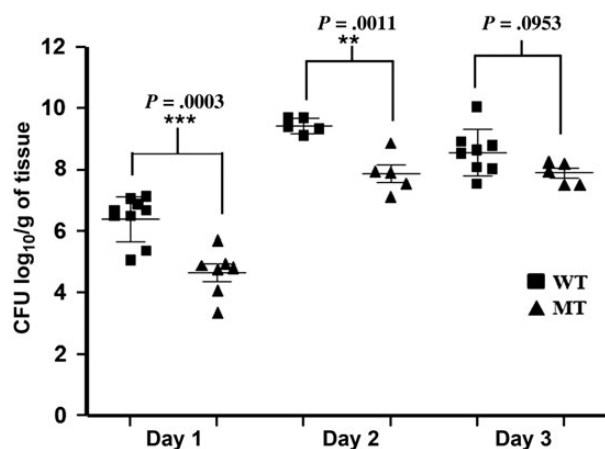

Figure 5. Bacterial burden in wild-type CYL11481 (WT) and its isogenic *mgr*A mutant (MT) in rat cardiac vegetations. Homogenized heart vegetation (100 μL) from each time point of the WT and *mgr*A mutant strains were quantitatively cultured onto tryptic soy agar plate and incubated at 37°C for 24 hours. Data represent colony-forming units (CFU) $\log_{10}$/g of tissue from 5 to 9 rats per time-point with *P* value. Statistical significance was analyzed by Student *t* test. ***Highly significant; **Significant.

S. aureus strain, CYL11481, had significantly higher vegetation bacterial counts than in animals infected with the *mgr*A mutant strain at both days 1 and 2 of infection. At day 3, the *mgr*A mutant strain had slightly lower bacterial counts than the parent strain but the difference was not statistically significant. These results suggest that MgrA plays an important role in virulence in the rat IE model.

DISCUSSION

The ability of *S. aureus* to cause a multitude of life-threatening human diseases suggests that the pathogenesis of *S. aureus* infections is highly complex. The expression of virulence genes is expected to be influenced by various host signals and thus likely to be different between in vivo and in vitro conditions. However, little is known about virulence gene regulation in vivo. In this study, we chose capsule as the model virulence factor in an in vivo virulence gene regulation study using a well-characterized rat IE model, because (1) regulation of capsule has been studied extensively in vitro; and (2) capsule production appears to be a key virulence factor [14, 15]. Our strategy was to correlate the temporal expression of this target virulence gene with that of its known regulators, during the infection course. We reasoned that the expression of the primary regulatory gene should correlate well with that of its target gene over time. To our knowledge, this is the first study of such temporal expression of a virulence gene, as well as its regulatory modulators in *S. aureus* in vivo. Our results suggest that, among all regulators tested, MgrA is the key regulator affecting capsule regulation in the rat IE model. This conclusion was further supported by the result that *mgr*A deletion abolished *cap*5 gene expression during the course of infection. This is in contrast to the in vitro results wherein Agr had the strongest effect on capsule expression. We have also previously shown that Agr strongly affected capsule over time in different growth phases in vitro [35]. Our results therefore suggest that capsule regulation is affected by host factors and regulated differently in vitro and in vivo; this supports the notion that *S. aureus* encounters different environmental signals in vivo than in vitro, and modulates its expression of virulence genes accordingly. The sharp increase in RNAIII expression in vivo at day 2 is noteworthy. The increase is consistent with a report that agr promoter activity in vegetations increased at 48 hours of infection in a rabbit IE model [36]. Interestingly, our results showed a drastic decrease of RNAIII at day 3. This spike in expression may indicate that Agr could still have an effect on capsule at that time point, which may explain the earlier study by van Wamel et al, who showed that the *cap*5 promoter in Newman was strongly affected by Agr in a rabbit IE model [32].

It is interesting that *clpC* expression in vivo was greatly reduced, especially in day 1 of infection. ClpC is an ATP-dependent

chaperone involved in protein quality control by refolding or degrading misfolded proteins [37]. The reduction may reflect a drastic change in physiology involving protein synthesis of the bacteria at an early stage of infection. In parental strain CYL11481, we found that ClpC promoted capsule by repressing *codY* expression in vitro. These results are opposite to the results in vivo where we found increased expression of *cap*5, but decreased expression of *clpC*, whereas *codY* remained largely unchanged. These results provide further evidence that regulation of gene expression is different between in vitro and in vivo conditions.

Detection of capsule in vivo depends on the specific animal models under study, and particularly the principal site of infection [14, 15, 32]. Production of CP8 has been shown to be increased in a rabbit IE model after 4 days of infection [38], consistent with our present study. However, paradoxically, capsule has been shown to attenuate *S. aureus* virulence possibly by masking adhesins required for colonization [39, 40]. How can capsule be induced in vivo, and yet attenuate virulence? One possible explanation is that *S. aureus* cells covered with capsule are less able to colonize the damaged heart tissue; however, after establishing colonization at such sites, induced capsule could then facilitate evasion of localized phagocytosis from host cells (eg, neutrophils [41–44]).

Our findings here that MgrA was the major activator of capsule in vivo suggests that MgrA may contribute to *S. aureus* resistance to phagocytosis and intracellular killing, in part, through its effect on capsule. Indeed, it was suggested that MgrA endows the bacteria with the ability to evade the host defense based on the finding that an *mgr*A mutation attenuated virulence in septic arthritis animal models [13]. Our results here also showed that *mgr*A mutation resulted in significant reduced bacterial burden by 1.5 to 2 log CFU/g vegetation at days 1 and 2 after infection. However, whether capsule is the key virulence factor through which MgrA affects virulence requires further studies.

Several regulators included in this study (Agr, Sae, MgrA, Arl, and CodY) have been shown to affect α-toxin expression in *S. aureus* [6, 18, 20, 16–30, 45, 46]. Agr activates *hla* through its effector RNAIII. The 5′ end of RNAIII directly interacts with the *hla* mRNA and unblocks the ribosome binding site of the *hla* gene [47], whereas the 3′ half of RNAIII inhibits *rot* translation by an antisense mechanism to relieve the repression of *hla* transcription by Rot [48, 49]. SaeR activates the transcription of *hla* by direct promoter binding [50], whereas MgrA activates *hla* transcription by binding to the promoter and by modulating Agr through SarS but not Sae [9]. The upstream region of the *hla* gene contains a CodY box suggesting that repression of *hla* transcription is by direct CodY binding. CodY can also repress *hla* via Agr [18, 20]. Although the results from these reports have been derived from different strains, they are largely consistent with our in vitro studies presented here using

CYL11481. However, we found Sae had the strongest effect at the transcriptional level, although Agr had the most effect on hemolytic activity, implying potential posttranscriptional regulation. The expression of *hla* in vivo has been studied in 2 reports. A study by Xiong et al [31] investigated the role of *agr*, *sar*A, and *sae* in controlling *hla* expression in *S. aureus* RN6390 and SH1000 backgrounds in a rabbit IE model; they found that *hla* expression in cardiac vegetations was moderately reduced in the *agr* and *sar*A single mutants, but was markedly reduced in the *sae* mutant [31]. In another study using a guinea pig device–related infection model, Goerke et al [27] demonstrated that Sae, but not Agr or SarA, was the key regulator of *hla* in vivo. These results are consistent with our finding that the *sae* expression correlated best with that of the *hla* in the rat IE model. Interestingly, we also found that the *mgr*A mutation in our study resulted in noticeable change in the *hla* and *rnaIII* expression patterns in the rat IE model, suggesting that MgrA has an effect, perhaps indirect, on both *hla* and *agr* expression in vivo.

In summary, the present investigation established the disparity of in vitro and in vivo regulation of capsule, and strongly underscored that MgrA is a major positive regulator of capsule gene expression in vivo in experimental *S. aureus* IE. The expression of the *hla* gene correlated best with the *saeR* gene expression in vivo, although MgrA may also have an effect on the regulation of *hla* gene expression.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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