3-2005

Rat/MgrA, a Regulator of Autolysis, Is a Regulator of Virulence Genes in Staphylococcus aureus

Susham Ingavale
Dartmouth College

Willem van Wamel
University of Utrecht

Thanh T. Luong
University of Kansas

Chia Y. Lee
University of Kansas

Ambrose L. Cheung
Dartmouth College

Follow this and additional works at: https://digitalcommons.dartmouth.edu/facoa

Part of the Medical Immunology Commons, Medical Microbiology Commons, and the Medical Pathology Commons

Recommended Citation
Ingavale, Susham; van Wamel, Willem; Luong, Thanh T.; Lee, Chia Y.; and Cheung, Ambrose L., "Rat/MgrA, a Regulator of Autolysis, Is a Regulator of Virulence Genes in Staphylococcus aureus" (2005). Open Dartmouth: Faculty Open Access Articles. 955.
https://digitalcommons.dartmouth.edu/facoa/955

This Article is brought to you for free and open access by Dartmouth Digital Commons. It has been accepted for inclusion in Open Dartmouth: Faculty Open Access Articles by an authorized administrator of Dartmouth Digital Commons. For more information, please contact dartmouthdigitalcommons@groups.dartmouth.edu.
Rat/MgrA, a Regulator of Autolysis, Is a Regulator of Virulence Genes in Staphylococcus aureus

Susham Ingavale, Willem van Wamel, Thanh T. Luong, Chia Y. Lee, and Ambrose L. Cheung*

Department of Microbiology, Dartmouth Medical School, Hanover, New Hampshire; Department of Medical Microbiology, University of Utrecht, Utrecht, The Netherlands; and Department of Microbiology, Molecular Genetics, and Immunology, University of Kansas Medical Center, Kansas City, Kansas

Received 10 August 2004/Returned for modification 8 October 2004/Accepted 12 November 2004

We have previously identified mgrA (rat) as a regulator of autolysis in Staphylococcus aureus. Besides its effect on autolytic activity, we recently found alterations in the expression of regulator and target virulence genes in the mgrA mutant. Northern analysis and transcription fusion assays showed that inactivation of mgrA has led to the downregulation of RNAIII of agr and hla and upregulation of sarS and spa. Although both SarA and agr are activators of α-hemolysin and a repressors of protein A synthesis, we found that the transcription of sarA was not affected in the mgrA mutant and vice versa, indicating that MgrA likely regulates hla and spa in a SarA-independent manner. Previously we have shown that SarT, a SarA homolog, is a repressor of hla and an activator of spa, presumably by activating SarS; however, analysis of the double sarT mgrA mutant for hla and spa transcription indicated that the mgrA-mediated effect is not mediated via sarT. Our results further demonstrated that the agr gene product regulates hla and spa expression in a dual fashion, with the first being agr dependent and the second agr independent. In the agr-independent pathway, MgrA binds directly to hla and the sarS promoter to modulate α-hemolysin and protein A expression. Thus, our studies here have defined the nature of interaction of mgrA with other regulators such as agr, sarS, and sarT and its role in regulating hla and spa transcription within the virulence regulatory network of S. aureus.

Staphylococcus aureus, a member of the family Micrococcaceae, is a gram-positive bacterium that normally colonizes the epithelial surface in 30 to 40% of humans. Despite advances in antimicrobial therapy, S. aureus remains a major cause of infections in the hospital setting. The spectrum of diseases caused by this organism is extremely wide, ranging from superficial skin infections to deep abscesses. Many of these infections begin locally (skin and catheters) and subsequently spread to the bloodstream, putting patients at risk of developing endocarditis and other metastatic complications. The capacity to cause a myriad of infections is probably attributable to the organism’s capacity to colonize and survive in diverse host niches during the infection process.

The pathogenicity of S. aureus is a complex process involving the spatial-temporal production of a diverse array of virulence factors. Many cell wall components that act as adhesins (e.g., fibrinogen and fibronectin binding proteins) or contribute to the evasion of host defense (protein A) are produced primarily during the exponential phase while the production of toxins and enzymes (alpha-hemolysin) that facilitate tissue invasion occurs postexponentially.

Adding to this complexity in S. aureus pathogenesis is the dramatic worldwide increase in antibiotic resistance among clinical isolates. More than 90% of staphylococcal isolates are now penicillin resistant. With the introduction of methicillin in the 1960s, the percentage of methicillin-resistant S. aureus infections has gradually increased, now up to 60 to 70% in the hospital setting. In the past few years, community-acquired methicillin-resistant S. aureus infections have been reported with increased frequency. The increased use of vancomycin, a glycopeptide antibiotic, has led to the emergence of vancomycin-resistant strains. This has raised the concern that resistant S. aureus infections may be difficult to treat with currently available antibiotics. Thus, there is a need to understand the pathogenetic process so that new molecular targets can be identified for the development of effective therapeutic agents.

The coordinated synthesis of cell wall proteins in the exponential phase and extracellular proteins during the postexponential phase suggests that many of these virulence determinants are governed by global regulatory elements. Members of these regulatory systems include the SarA protein family and a number of two-component regulatory systems such as AgrAC, SaeRS, LytRS, ArfRS, SrrAB, YycFG, and VraRS. The agr and sar loci comprise two critical global regulatory elements that coordinate synthesis of cell wall and extracellular virulence proteins during the exponential and postexponential phases, respectively.

The agr locus encodes two divergent transcripts, RNAII and RNAIII, driven by two distinct promoters, P2 and P3, respectively. The P2 transcript encodes four genes, agrB, agrD, agrC, and agrA, comprising a two-component quorum-sensing system normally required for the activation of RNAII and RNAIII. The RNAII gene product AgrD encodes a 46-residue peptide which is processed and secreted as an autoinducing peptide with the aid of the putative membrane component AgrB. This cyclic peptide carries a quorum-sensing...
participate in the growth cycle (1, 12), a number of SarA homologs have been found to be involved in the expression of a variety of virulence proteins. SarA is an important regulator of virulence determinants in S. aureus. MgrA, SarT, and SarU are involved in regulating RNAIII transcription (49), SarS, an activator of protein A synthesis (10, 52), and SarU, which activates RNAIII transcription (33). Remarkably, SarT was found to activate sarS expression (48), indicating that agr may repress protein A expression by down-regulating sarT and subsequently sarS.

In this study, we report the interaction of Rat/MgrA protein with agr and its effect on the sarS and hla promoters, culminating in the expression of α-hemolysin and protein A. A mutation in mgra resulted in altered expression of RNAIII, sarS, hla, and spa expression. We also provided additional evidence that mgra has a dual role in regulating hla and spa expression. In the agr-dependent pathway, decreased agr transcription in mgra mutants would lead to reduced hla transcription and an increase in spa transcription. In the agr-independent pathway, direct interactions of MgrA and hla with the sarS promoter resulted in activation or repression of the respective gene. Based on these results, we propose MgrA to be an important regulator of virulence determinants in S. aureus.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. Phages Φ11 and 80a were used as transducing phages for S. aureus strains. S. aureus cells were grown at 37°C with aeration in CYGBP 03GL broth (39) or tryptic soy broth supplemented with antibiotics as indicated. Luria-Bertani (LB) was used for cultivating *Escherichia coli.*

### TABLE 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Comment</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. aureus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RN2420</td>
<td>Mutant strain of 8325-4 that accepts foreign DNA</td>
<td>39</td>
</tr>
<tr>
<td>RN6390</td>
<td>agr&lt;sup&gt;-&lt;/sup&gt; laboratory strain related to 8325-4, maintains hemolytic pattern when propagated on sheep erythrocytes</td>
<td>39</td>
</tr>
<tr>
<td>COL</td>
<td>Meticillin-resistant laboratory strain</td>
<td>54</td>
</tr>
<tr>
<td>Newman</td>
<td>Laboratory strain</td>
<td>37</td>
</tr>
<tr>
<td>FDA486</td>
<td>Wild-type strain FDA486, sigB&lt;sup&gt;+&lt;/sup&gt;, intact rsbU</td>
<td>43</td>
</tr>
<tr>
<td>RN6911</td>
<td>agr mutant of RN6390 (Δagr::tetM)</td>
<td>42</td>
</tr>
<tr>
<td>ALC3043</td>
<td>sarT mutant of RN6390 (ΔsarT::tetK)</td>
<td>This study</td>
</tr>
<tr>
<td>ALC2530</td>
<td>mgrA mutant of RN6390 (ΔmgrA::ermC)</td>
<td>This study</td>
</tr>
<tr>
<td>ALC2531</td>
<td>ALC2530 complemented with the mgrA gene in single copy</td>
<td>This study</td>
</tr>
<tr>
<td>ALC2542</td>
<td>mgrA mutant of Newman (ΔmgrA::ermC)</td>
<td>This study</td>
</tr>
<tr>
<td>ALC2547</td>
<td>mgrA mutant of Newman (ΔmgrA::ermC)</td>
<td>This study</td>
</tr>
<tr>
<td>ALC3632</td>
<td>mgrA mutant of FDA486 (ΔmgrA::ermC)</td>
<td>This study</td>
</tr>
<tr>
<td>ALC2537</td>
<td>agr mgrA (deletion) double mutant of RN6390</td>
<td>This study</td>
</tr>
<tr>
<td>ALC3046</td>
<td>sarT mgrA (deletion) double mutant of RN6390</td>
<td>This study</td>
</tr>
<tr>
<td>ALC3188</td>
<td>agr sarT (deletion) double mutant</td>
<td>This study</td>
</tr>
<tr>
<td>ALC3191</td>
<td>mgrA sarT agr (deletion) triple mutant</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XL-1 Blue</td>
<td>General-purpose host strain for cloning</td>
<td>31</td>
</tr>
<tr>
<td>Inv&lt;sup&gt;o&lt;/sup&gt;F&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Host strain for the TA cloning vector (pCR2.1)</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Comment</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCR2.1</td>
<td>E. coli PCR cloning vector</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pALC1484</td>
<td>Derivative of pSK236 containing the promoterless gfpuvr gene</td>
<td>25</td>
</tr>
<tr>
<td>pALC1740</td>
<td>pALC1484 with hla promoter fragment</td>
<td>27</td>
</tr>
<tr>
<td>pALC1741</td>
<td>pALC1484 with spa promoter fragment</td>
<td>27</td>
</tr>
<tr>
<td>pALC1743</td>
<td>pALC1484 with P3 agr promoter fragment</td>
<td>25</td>
</tr>
<tr>
<td>pALC1793</td>
<td>pALC1484 with sarS promoter fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pALC1594</td>
<td>235-bp hla promoter region in pCR2.1</td>
<td>13</td>
</tr>
<tr>
<td>pALC2321</td>
<td>sarS promoter region in pCR2.1</td>
<td>46</td>
</tr>
<tr>
<td>pALC1996</td>
<td>agr P2-P3 promoter region in pALC1484</td>
<td>14</td>
</tr>
<tr>
<td>pRN6735</td>
<td>Contains promoterless RNAIII under control of the hlaZ promoter</td>
<td>26</td>
</tr>
</tbody>
</table>

function and, upon reaching the threshold level, binds to AgrC, the membrane sensor component of a two-component system, eventually activating the response regulator AgrA (24, 23). Presumably, phosphorylated AgrA would bind to the agr P3 promoter to promote transcription from the P3 promoter.

The P3 transcript, designated RNAIII, is the regulatory molecule of agr and acts on target genes mainly at the level of transcription and, to a lesser extent, translation (42, 22). Once RNAIII is synthesized, it somehow upregulates the transcription of exoprotein genes (e.g., *hla*) while downregulating genes encoding cell wall proteins (e.g., *spa*, encoding protein A, and *fnb*, encoding fibronectin binding protein) (22). However, the exact manner by which RNAIII activates target gene transcription is not clearly defined.

The agr locus is also activated by SarA, which binds to the agr P2-P3 promoter region to activate RNAII and RNAIII transcription (9, 13). The sarA locus is composed of three overlapping transcripts, *sarA* P1, P3, and P2, each encoding the 14.5-kDa SarA protein. GeneChip analysis indicated that the *sarA* locus affects the transcription of ~120 genes in *S. aureus* (2, 15). While the *sarA* and *agr* loci are the major controlling elements for the expression of a variety of virulence proteins during the growth cycle (1, 12), a number of SarA homologs (members of the SarA protein family) have been found to participate in the *sarA* and *agr* regulatory cascade (12, 5), including SarT, a repressor of *hla* which is normally repressed by SarA and *agr* (49), SarS, an activator of protein A synthesis (10, 52), and SarU, which activates RNAIII transcription (33). Remarkably, SarT was found to activate sarS expression (48), indicating that *agr* may repress protein A expression by down-regulating *sarT* and subsequently *sarS*. In this study, we report the interaction of Rat/MgrA protein with *agr* and its effect on the *sarS* and *hla* promoters, culminating in the expression of α-hemolysin and protein A. A mutation in *mgra* resulted in altered expression of RNAIII, *sarS*, *hla*, and *spa* expression. We also provided additional evidence that *mgra* has a dual role in regulating *hla* and *spa* expression. In the *agr*-dependent pathway, decreased *agr* transcription in *mgra* mutants would lead to reduced *hla* transcription and an increase in *spa* transcription. In the *agr*-independent pathway, direct interactions of MgrA and *hla* with the *sarS* promoter resulted in activation or repression of the respective gene. Based on these results, we propose MgrA to be an important regulator of virulence determinants in *S. aureus*. 
coli. Antibiotics used for S. aureus were erythromycin, 5 μg/ml; tetracycline, 3 μg/ml; chloramphenicol, 10 μg/ml; and minocycline, 2.5 μg/ml. For E. coli, the following concentrations were used: ampicillin, 50 μg/ml; and spectinomycin, 75 μg/ml.

Genetic manipulations in E. coli and S. aureus. For the propagation of all plasmid constructs, E. coli strain DH5α was used. Standard molecular biology and recombinant DNA techniques were followed (31). S. aureus RN2240, a restriction-deficient derivative of strain 8325-4 (39), was used as the initial recipient for the transformation of plasmid constructs by electroporation (47).

The construction of the mgra (mut) deletion mutant in the RN6390 background to yield ALC2530 has been previously described (21). To generate mgra deletion mutants in other genetic backgrounds, we used 411 and 80a phage lysates of ALC2530 to infect strains COL, Newman, FDA486, RN6911 (mgra mutant), and ALC3943 (sarT mutant) (Table 1). For the construction of the sarT agr double mutant (ALC3188), a 411 phage lysate of the sarT mutant was used to infect the agr mutant RN6911. Similarly the triple mgra agr sarT mutant (ALC3191) was constructed by infecting the mgra sarT double mutant with a phage lysate of the RN6911 agr mutant.

Isolation of RNA and Northern blot hybridization. Overnight cultures of S. aureus were diluted 1:100 in CYGP and grown to late log (optical density at 650 nm [OD600] = 1.2) phase. The cells were harvested and processed with a Trizol isolation kit (Gibco BRL, Gaithersburg, Md.) in combination with 0.1-mm silica-silica beads in a Biospec reciprocating shaker to yield RNA as described (7). 15 μg of each sample was electrophoresed in a 1.5% agarose-0.66 M formaldehyde gel in morpholinepropanesulfonic acid (MOPS) running buffer (20 mM MOPS, 10 mM sodium acetate, 2 mM EDTA, pH 7.0). Blotting of RNA onto nylon membranes (Amersham, Arlington Heights, Ill.) was performed with the Turboblotter alkaline transfer system (Schleicher & Schuell, Keene, N.H.). For detection of specific transcripts (RNAIII, sarT, spa, hla, and mgra), gel-purified DNA probes were radiolabeled with [α-32P]ATP by using T4 polynucleotide kinase. Similarly, a 228-bp fragment representing a region between the P2 and P3 promoters (nucleotides 125169 to 125432) (10) obtained from plasmid pALC1484 (pSK236-based plasmid containing the promoterless gfpuvr gene) upstream of the gfpuvr gene to generate transcriptional fusions. Restriction analysis and DNA sequencing confirmed the orientation and authenticity of the promoter fragments. The recombinant plasmids containing these promoters were first introduced into S. aureus strain RN4220 by electroporation (47). Plasmids purified from RN4220 transformants were then electroporated into RN6390 and its isogenic mgra and other mutants.

After overnight culture, S. aureus strains harboring the recombinant plasmids were diluted 1:100 and grown at 37°C with shaking in tryptic soy broth with chloramphenicol (10 μg/ml). Aliquots (200 μl) were transferred hourly to microtiter plates to assay for cell density (OD600) and fluorescence for 10 h in a FL600 fluorescence spectrophotometer (BioTek Instrument, Winooski, Vt.). Promoter activities were plotted as mean fluorescence/OD600 ratio to minimize the effect of bacterial concentration, confirmed the Northern analysis data (Fig. 1A and 1C).

Transcriptional fusion studies of different promoters linked to the gfpuvr reporter gene. To confirm the effect of the mgra mutation on promoter activities of other regulators (RNAIII and sarT) and target genes such as hla and spa, we cloned promoter fragments of these genes into the shuttle vector pALC1484 (pSK236-based plasmid containing the promoterless gfpuvr gene) upstream of the gfpuvr gene to generate transcriptional fusions. Restriction analysis and DNA sequencing confirmed the orientation and authenticity of the promoter fragments. The recombinant plasmids containing these promoters were first introduced into S. aureus strain RN4220 by electroporation (47). Plasmids purified from RN4220 transformants were then electroporated into RN6390 and its isogenic mgra and other mutants.

Gel shift assays. The purification of Mgra (Rat) protein has been described (21). To determine if the recombinant Mgra protein binds to the agr and hla promoters, a 228-bp fragment representing a region between the P2 and P3 promoters of agr (from nucleotides 1528 to 1756) (26) and a 253-bp hla promoter fragment (nucleotides 1 to 80 plus 155 bp upstream of the start codon) (13) were end-labeled with [γ-32P]ATP by using T4 polynucleotide kinase. Similarly, a 264-bp sarT promoter fragment (nucleotides 125169 to 125432) (10) obtained from plasmid pALC2321 (Table 1) was end-labeled. Labeled fragments (0.1 ng) were incubated at room temperature for 20 min with various amounts of purified Mgra protein in 25 μl of binding buffer (25 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 75 mM NaCl, 1 mM dithiothreitol, and 10% glycerol) containing 0.5 μg of calf thymus DNA per ml. The reaction mixtures were analyzed in a 6.0% nondenaturing polyacrylamide gel. The band shifts were detected by exposing dried gels to X-ray film.

RESULTS

Effect of the mgra mutation on genes involved in virulence. In an earlier study, we have reported that Mgra (Rat) is a regulator of autolysis that shares sequence similarity with members of the SarA and MarR families (21). As SarA is part of the complex regulatory network that modulates expression of other regulators as well as target virulence genes, we wanted to determine if Mgra constitutes part of this network by examining the effect of the mgra mutation on known regulators of virulence determinants.

We have focused on major regulators such as agr, sar, and sarT, and members of the SarA protein family (SarA, SarT, and SarS) that are involved in the control of the α-hemolysin (hla) and protein A (spa) genes, two putative virulence genes in S. aureus.

We used the ALC2530 strain as the background for the transformation of the plasmid constructs. To confirm the effect of the mgra mutation on the RNAIII and sarT gene expression, we transformed strain RN6390 and its isogenic mgra mutant with shuttle plasmids carrying the gfpuvr reporter gene driven by the RNAIII, sarT, hla, and spa promoters. Green fluorescent protein (GFP) expression levels in the mutant, expressed as fluorescence units per OD unit to minimize the effect of bacterial concentration, confirmed the Northern analysis data (Fig. 1A and 1C).

To confirm the effects of the mgra mutation on RNAIII, sarT, hla, and spa transcription, we transformed strain RN6390 and its isogenic mgra mutant with plasmids carrying the gfpuvr reporter gene driven by the RNAIII, sarT, hla, and spa promoters. Green fluorescent protein (GFP) expression levels in the mutant, expressed as fluorescence units per OD unit to minimize the effect of bacterial concentration, confirmed the Northern analysis data (Fig. 1A and 1C).

To ensure that these observations are not unique to the mgra mutation in the RN6390 background, we also ascertained the presence of RNAIII, hla, and spa transcripts in other mgra mutants of strains COL, Newman, and FDA486. Our results indicated that the mgra mutants in all these genetic backgrounds displayed decreased levels of RNAIII and hla compared with the parent, while spa levels were increased (data not shown). Taken together, these results showed that the mgra gene product modulates virulence gene expression by acting as a positive regulator for RNAIII and hla and a negative regulator of spa.

Regulation of hla expression. Previous studies from our laboratory have shown that SarA and RNAIII of agr could activate hla expression directly, while SarA also modulates hla transcription by repressing sarT (13, 49). Since Mgra positively affects the expression of both agr (RNAIII) and hla, we wanted to explore if this effect on hla by mgra could be direct and/or indirect via agr or the agr sarT pathway. To dissect these possibilities, a panel of double and triple deletion mutant strains were constructed in strains RN6390 and Newman, including mgra sarT, mgra agr, sarT agr, and mgra sarT agr mutants (described in Materials and Methods). All the mutants were
RNA was harvested from cells grown to an OD650 of
scripts in RN6390 and isogenic
earlier points. These experiments were repeated at least three times
1426 INGAVALE ET AL. INFECT. IMMUN.

The probe for RNAIII was a 920-bp fragment containing the
hld
RNAIII and
sarS
agr
representing late exponential phase. C. Northern blot analysis of
involved in virulence. A. Northern blot analysis of
hla

and
spa
promoters in RN6390 and the
mgrA
mutant was also measured. Promoter activation was plotted as mean
fluorescence/OD650 ratio, using average values of triplicate readings at an OD 650 of

1.4. Comparable differences were also observed at earlier points. These
results implied that MgrA could control hla expression both directly and indirectly via
agr.

Assessing hla expression by providing RNAIII in trans. To
further validate the role of agr in hla expression in
mgrA
mutants, we elected to complement the
mgrA
mutant with a plasmid carrying RNAIII (pRN6735). The cloned RNAIII fragment was under the control of a β-lactamase (blaZ) promoter and constitutively expressed in the absence of pL524, the plasmid that carried the β-lactamase repressor (26). Plasmid RN6735 was used to transform parental strains RN6390 and Newman and isogenic
mgrA
and
agr
mutants. Restriction analysis confirmed the presence of plasmid RN6735 in the resultant transformants. These transformants were then analyzed by
confirmed by PCR and Southern blots. These strains were then analyzed for RNAIII and hla expression.

As expected, the mRNA levels of RNAIII were not readily detectable in the
mgrA
mutant and
agr
mutant, while RNAIII expression in the
sarT
mutant was similar to that in the parental strain RN6390 (Fig. 2A). Analogous to the
mgrA
and
agr
mutants, the remaining double and triple mutants did not express RNAIII well. Interestingly, the
mgrA
mutant, coinciding with its low
agr
expression, expressed hla to a level similar to that of the
agr
mutant. In the
mgrA
sarT
double mutant, the
hla
transcript level, concordant with a low level of RNAIII, remained undetectable, indicating that activation of hla by
mgrA
is not mediated via
sarT
(Fig. 2A and 2B). In the
mgrA
agr
double mutant, hla expression, similar to that of the single
mgrA
and
agr
mutants, was significantly lower than that of the parent (Fig. 2A and 2B). As expected, the expression of hla in the triple
mgrA
sarT
agr
mutant remained low and not readily detectable (Fig. 2A and 2B).

GFP fusion assays also confirmed these results (Fig. 2A and 2B), leading us to hypothesize that
mgrA regulates hla indirectly via
agr
or directly on hla or both and that there is very little cross talk, if any, between
mgrA
and
sarT
for hla expression.

Gel shift assay. By virtue of its homology with members of the SarA and MarR protein families, we speculate that MgrA might also be a DNA-binding protein (21). Given that an
mgrA
mutation resulted in downregulation of RNAIII and hla, we wanted to explore if MgrA binds to these promoters to modulate gene transcription. Using purified MgrA protein and end-labeled hla promoter fragment (~1 ng), we found that MgrA could bind to the 235-bp hla promoter fragment with 1 μg of protein (Fig. 3A), while a nearly complete shift occurred in the presence of 2.0 μg of MgrA. The protein-DNA complex could be disrupted with a 50-fold excess of unlabeled hla promoter fragment (lane 7 in Fig. 3A) but not with a 160-bp spa promoter fragment (Fig. 3A, lane 9) or a nonspecific competitor such as a 300-bp asp23 promoter fragment at comparable concentrations (Fig. 3A, lane 8).

Likewise, the mobility of the 228-bp end-labeled
agr
P2-P3 promoter fragment was also retarded by MgrA, with the retardation taking place at 1.5 μg of protein (Fig. 3B). As with the hla promoter fragment, unlabeled
agr
promoter fragment competed successfully for the binding of MgrA, whereas the spa and asp23 promoter fragments did not (Fig. 3B). Collectively, these data demonstrated that MgrA likely regulates hla and RNAIII by interacting directly with the promoters. As RNAIII of
agr
is known to regulate hla transcriptionally (42), these results implied that MgrA could control hla expression both directly and indirectly via
agr.

FIG. 1. Effect of the
mgrA
mutation on the expression of genes involved in virulence. A. Northern blot analysis of
hla
and
spa
transcripts in RN6390 and isogenic
mgrA
mutant ALC2530. The open reading frame of each gene was used as a probe for the Northern blots. RNA was harvested from cells grown to an OD650 of ~1.2, representing late exponential phase. The expression of GFP driven by the
hla
and
spa
promoters in RN6390 and the
mgrA
mutant was also measured. Promoter activation was plotted as mean fluorescence/OD650 ratio, using average values of triplicate readings at an OD 650 of ~1.4. Comparable differences were also observed at earlier points. These experiments were repeated at least three times with similar results. B, RN6390; C. Northern blot analysis of
sarA
and
sarS
transcripts in RN6390 and
mgrA
mutant ALC2530. The open reading frame of each gene was used as a probe for the Northern blots. RNA was harvested from cells grown to an OD650 of ~1.2, representing late exponential phase. C. Northern blot analysis of
agr
RNAIII and
sarS
transcripts in RN6390 and
mgrA
mutant ALC2530. The probe for RNAIII was a 920-bp fragment containing the
hld
gene. The open reading frame of the
sarS
gene was used as a probe for the Northern blot. RNA was harvested from cells grown to an OD650 of ~1.2, representing late exponential phase. The expression of GFP driven by the
agr
RNAIII and
sarS
promoters in RN6390 and the
mgrA
mutant was also measured. Promoter activation was plotted as mean fluorescence/OD650 ratio, using average values of triplicate readings at an OD 650 of ~1.4. Comparable differences were also observed at earlier points. These experiments were repeated at least three times with similar results. (RN6390; C, 
agr
mutant ALC2530).
Northern blotting to determine the levels of RNAIII and hla expression.

Our results indicated that RNAIII, as driven by the blaZ promoter, was expressed constitutively in the wild-type strain as well as in the mgrA and agr mutants (Fig. 4). Despite the presence of RNAIII in induced mgrA and parental strains, the expression of hla, while activated, was lower in the mgrA mutants than in the parents in both the RN6390 and Newman backgrounds (lanes 2 and 4 and lanes 6 and 8 in Fig. 4). Collectively, gel shift and transcriptional data with RNAIII provided in trans showed that mgrA-regulation of hla is likely under bimodal control, with both direct (hla promoter) and indirect (agr promoter) regulation.

Regulation of protein A by mgrA is dependent on sarS. As described earlier (Fig. 1A), mgrA also modulates protein A expression negatively. Previously, the expression of spa has been shown to be positively regulated by sarS (52, 10). Additional gel shift and transcriptional studies revealed that sarS is positively controlled in part by sarT and negatively controlled by agr (48, 33). As the mgrA locus positively controls agr, we wanted to ascertain if mgrA also represses sarS and spa expression via agr. To ascertain this pathway, we used the same set of strains that we used to dissect the regulation of hla.

Northern blotting data revealed that the sarS transcript level, as expected, was not readily detectable in the parent but was increased in the mgrA mutant (Fig. 5A). Interestingly, the
Interestingly, in the double
mutations were confirmed by promoter fusion assays (Fig. 5B).

gesting that the effect of
mgrA
Likewise, in the
mgrA agr
sarT agr
mutant, the
sarS
there was little if any
sarS
than those of
agr
elevated. In contrast, the effect of
sarT agr
in the
sarS
mutant ALC3043;

FIG. 5. Transcription of sarS in RN6390 and isogenic mutants. A.
Northern blot analysis of the sarS transcript in RN6390 and isogenic mutants. The open reading frame of sarS was used as a probe. RNA was harvested from cells grown to an OD_{650} of ~1.2, representing late exponential phase. B. The expression of GFP driven by the sarS promoter was also measured. Promoter activation was plotted as mean fluorescence/OD_{650} ratio, using average values of triplicate readings at an OD_{650} of ~1.4. This experiment was repeated at least three times with similar results. □, RN6390; □, mgrA mutant ALC2530; ■, sarT mutant ALC3043; □, agr mutant RN6911; □, sarT mgrA mutant ALC3046; □, agr mgrA mutant ALC2537; ■, agr sarT mutant ALC3188; and □, mgrA sarT agr mutant ALC3191.

increase in sarS promoter activity, as assessed by transcriptional fusions, was higher in the mgrA mutant than in the agr mutant, implying an additional effect on sarS by mgrA other than those of agr (Fig. 5B). As expected in the absence of sarT, there was little if any sarS transcription (48). These observations were confirmed by promoter fusion assays (Fig. 5B). Interestingly, in the double mgrA sarT mutant and triple mgrA sarT agr mutant, the sarS transcript level remained high, suggesting that the effect of mgrA on sarS is not mediated via sarT. Likewise, in the mgrA agr mutant, sarS levels continued to be elevated. In contrast, the effect of agr on sarS is sarT dependent, as exemplified by the near absence of sarS transcription in the sarT agr mutant. Collectively, these results indicate that modulation of sarS expression by mgrA is not mediated via sarT and is only partially dependent on agr.

To correlate sarS to spa expression, we also analyzed spa expression in the above mutants. Our results indicated that the spa mRNA level in all these strains corresponded quite nicely with the sarS levels (Fig. 6A), with upregulation in the mgrA, mgrA sarT, mgrA agr, and mgrA agr sarT mutants and down-regulation or relatively unaltered levels in sarT and agr sarT mutants, using both Northern and transcriptional fusion assays (Fig. 6A and B).

The observation that mgrA may impact on sarS independent of agr and sarT led us to speculate that a direct interaction between MgrA and the sarS promoter may occur. Gel shift assays with labeled sarS promoter (240 bp) and purified MgrA protein showed that 750 ng of MgrA was required to retard the mobility of the sarS promoter (Fig. 7). At 2,000 ng, there was a complete shift of the promoter DNA in the gel shift assay. The protein-DNA complex could be disrupted by 50-fold excess unlabeled promoter DNA (Fig. 7) while the nonspecific promoter DNA fragments, including the spa and asp23 promoters, did not alter the binding of MgrA to the sarS promoter (Fig. 7). Seen together, these data showed that MgrA likely acts as a negative regulator of spa by repressing sarS expression.

FIG. 7. Gel shift assays of purified MgrA with sarS promoter fragment. MgrA protein in increasing concentrations was incubated with the end-labeled sarS promoter fragment. In competition assays, MgrA protein (1,000 ng) was incubated with end-labeled promoter in the presence of a 50-fold excess of unlabeled specific competitor (sarS) (lane 7) or the nonspecific competitor (~300-bp asp23 promoter in lane 8 and the ~160-bp spa promoter fragment in lane 9).
To assess if the absence of RNAIII could account for the overexpression of sarS and hence spa in the mgrA mutant, we also provided RNAIII in trans with a plasmid containing the RNAIII sequence in these strains. The strains were then evaluated for spa expression by Northern blotting. Our results indicated that overexpression of RNAIII under the β-lactamase promoter led to significant repression of spa in the mgrA mutants of strains RN6390 and Newman compared with the mgrA mutant controls (Fig. 8). These data imply that MgrA likely has a dual role in regulating spa expression, with the first pathway dependent on agr, wherein an absence of RNAIII in an mgrA mutant leads to an increase in sarS and ultimately spa expression. In the second pathway, a direct interaction between MgrA and the sarS promoter likely occurs to repress sarS transcription.

**DISCUSSION**

With the advance of genomic information coupled with transcriptional analysis, it is now recognized that a complex regulatory network exists to control growth phase-dependent expression of a number of virulence determinants (1, 15, 40). The open reading frame of the spa gene was used as a probe. RNA was harvested from cells grown to an OD_600 of ~1.2, representing late exponential phase.

Analysis of the double sarT agrC mutant (ALC3046) for hla transcription indicated that the mgrA-mediated effect did not occur via sarT. Likewise, this effect is also independent of sae, as sae transcription was unaltered in the mgrA mutant (Fig. 1B). While it would seem that reduced expression of RNAIII in the mgrA mutant would account for the downmodulation in hla expression, our finding that the hla transcript level in the mgrA agr double mutant (ALC2537) was lower than in the agr mutant (Fig. 2B) would suggest a dual effect of mgrA on hla. The dual mode of regulatory control by mgrA on hla was confirmed by gel shift assays (Fig. 3) as well as transcriptional analysis of the mgrA mutant with RNAIII provided in trans (Fig. 4). Thus, the mgrA gene product acts as a positive regulator for agr to promote hla transcription as well as binds to the hla promoter to augment gene transcription.

In a previous study, Luong et al (30) also discovered mgrA in a Tn917 transposon screen, searching for mutants that had altered capsular production in capB strain Becker. Contrary to our finding here, they reported upregulation of alpha toxin and protein A synthesis in an mgrA mutant of strain Becker; unfortunately, the impact of mgrA on the expression of agr RNAIII was not evaluated in detail in that study. Because of this discrepancy, we sought to analyze RNAIII and hla expression in isogenic mgrA strains in four different genetic backgrounds, including RN6390, Newman, COL, and FDA486. All of the aforementioned mgrA mutants exhibited reduced levels of agr and hla expression compared with their parental counterparts. We have also analyzed hla transcription in the mgrA mutant of Becker and found no significant increase in the transcript level compared with the parental strain; transcriptional fusion studies with the hla promoter also confirmed the Northern blot results (data not shown). In distinction to our transcriptional approach, Luong et al. studied the phenotypic effect of mgrA on alpha-toxin production by hemolytic assays on blood agar plates. Whether altered levels of V8 protease as reported in the original mgrA mutant (21) account for the increase in alpha-toxin in the Becker strain is not clear. However, in subsequent experiments in which Luong et al. transduced the mgrA mutation from strain Becker to Newman, they also found reduced levels of α-hemolysin production and elevated levels of protein A synthesis (unpublished data). Thus, differences in the genetic background may have accounted for the divergent results. Although we previously were able to
FIG. 9. Model for MgrA-mediated bimodal regulation of virulence genes in *S. aureus*. MgrA upregulates expression of RNAIII. RNAIII activates *hla* expression and downregulates *sarS*, leading to repression of *spa*. Besides the *agr*-dependent pathway, MgrA binds directly to the *hla* promoter to augment its activation. Similarly, MgrA binds to the *sarS* promoter to downregulate *spa* expression.

complement the *mgrA* mutation in *trans* (21), we could not entirely rule out a subtle polar effect of the mutation on neighboring genes that may have altered *hla* translation but not transcription. Studies are now in progress to assess if the *mgrA* mutation may have affected neighboring genes both upstream and downstream of the *mgrA* mutation.

Previous studies from our laboratory have shown that SarT positively modulates *sarS* expression, which, in turn, upregulates *spa* expression (48). Although the transcript levels of *sarS* and *spa* were increased in the *mgrA* mutant (Fig. 5 and 6), we discovered that *sarT* did not play a major role in *spa* regulation in *mgr*− strains, similar to the pattern we observed for *hla* regulation. However, given that the *sarS* transcript level in the *mgrA* mutant was higher than that of the *agr* mutant (Fig. 5B), we speculate that the effect of *mgrA* on *spa* is likely to be both *agr* dependent and *agr* independent. The dependency on *agr* was confirmed by the repression of *spa* transcription in an *mgrA* mutant with RNAIII provided in *trans* (Fig. 8). Additionally, the finding that MgrA binds directly to the *sarS* promoter but not the *spa* promoter (last lanes in Fig. 3A, 3B, and 7) is consistent with a direct effect of MgrA on *sarS* to downmodulate *spa* expression. These data thus demonstrated bimodal regulation of protein A by the *mgrA* gene product.

From these data, we propose that *mgrA*, similar to other members within the single-and double-domain SarA protein subfamilies, is an important global regulator of virulence determinants in *S. aureus*. We propose (Fig. 9) that the *mgrA* gene product likely regulates *hla* and *spa* expression bimodally, with the first mode being *agr*-dependent and the second mode *agr* independent. Interestingly, the upregulatory effect of *mgrA* on *hla* does not rely on repression of *sarT*, while its effect on *spa* is dependent on *sarS* and not *sarT* despite the known positive effect of *sarT* on *sarS* (48, 49).

In our previous study, we described *mgrA* as a negative regulator of autolysis. Concomitantly, two other groups have described *mgrA* as a positive regulator of capsular polysaccharide synthesis (30) and possibly of *norA*, which encodes an efflux pump for mediating fluoroquinolone resistance (53). We have now added to this spectrum of effects the ability of *mgrA* to positively regulate *agr* as well as its direct effect on *hla* and *sarS*, eventually yielding what appears to be an *agr*-negative phenotype in an *mgrA* mutant. Given that *mgrA* is a negative regulator of autolysis and a positive regulator of *agr*, it seems logical that interference with MgrA may be a reasonable antiinfective strategy, since this approach would promote autolysis while minimizing the expression of *agr*, an important regulator of virulence determinants in *S. aureus*.

**ACKNOWLEDGMENTS**

This work was supported by research grants AI47441 (A.L.C.) and AI54607 (C.Y.L.) from the NIH.

**REFERENCES**

Mgra regulates virulence genes


