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

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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 *mgrA* 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 (29). 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 (45). 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 (45).

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 (20). The increased use of vancomycin, a glycopeptide antibiotic, has led to the emergence of vancomycin-resistant strains (4, 50). 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 (12). Members of these regulatory systems include the SarA protein family (8, 12) and a number of two-component regulatory systems such as AgrAC (26), SaeRS (41, 19, 51), LytRS (3), ArlRS (16), SrrAB (55), (44), YycFG (36), and VraRS (28). The *sarA* and *agr* loci comprise two critical global regulatory elements that coordinate synthesis of cell wall and extracellular virulence proteins during the exponential and postexponential phases, respectively (1, 12).

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 (38, 42). 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

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TABLE 1. Strains and plasmids

Strain or plasmid	Comment	Reference or source
S. aureus		
RN4220	Mutant strain of 8325-4 that accepts foreign DNA	39
RN6390	*agr*$^+$ laboratory strain related to 8325-4, maintains hemolytic pattern when propagated on sheep erythrocytes	39
COL	Methicillin-resistant laboratory strain	54
Newman	Laboratory strain	37
FDA486	Wild-type strain FDA486, *sigB*$^+$, intact *rsbU*	43
RN6911	*agr* mutant of RN6390 (Δ*agr*::*tetM*)	42
ALC3043	*sarT* mutant of RN6390 (Δ*sarT*::*tetK*)	This study
ALC2530	*mgrA* mutant of RN6390 (Δ*mgrA*::*ermC*)	21
ALC2531	ALC2530 complemented with the *mgrA* gene in single copy	21
ALC2542	*mgrA* mutant of COL (Δ*mgrA*::*ermC*)	This study
ALC2547	*mgrA* mutant of Newman (Δ*mgrA*::*ermC*)	This study
ALC3632	*mgrA* mutant of FDA486 (Δ*mgrA*::*ermC*)	This study
ALC2537	*agr mgrA* (deletion) double mutant of RN6390	21
ALC3046	*sarT mgrA* (deletion) double mutant of RN6390	This study
ALC3188	*agr sarT* (deletion) double mutant	This study
ALC3191	*mgrA sarT agr* (deletion) triple mutant	This study
E. coli		
XL-1 Blue	General-purpose host strain for cloning	31
InvαF′	Host strain for the TA cloning vector (pCR2.1)	Invitrogen
Plasmids		
pCR2.1	*E. coli* PCR cloning vector	Invitrogen
pALC1484	Derivative of pSK236 containing the promoterless gfp_{uvr} gene	25
pALC1740	pALC1484 with *hla* promoter fragment	27
pALC1741	pALC1484 with *spa* promoter fragment	27
pALC1743	pALC1484 with P3 *agr* promoter fragment	25
pALC3179	pALC1484 with *sarS* promoter fragment	This study
pALC1594	235-bp *hla* promoter region in pCR2.1	13
pALC2321	*sarS* promoter region in pCR2.1	46
pALC1996	*agr* P2–P3 promoter region in pALC1484	14
pRN6735	Contains promoterless RNAIII under control of the *blaZ* promoter	26

function and, upon reaching the threshold level, binds to AgrC, the membrane sensor component of a two-component system, eventuating in the activation of 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 transcriptions (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 downregulating *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 80α were used as transducing phages for *S. aureus* strains. *S. aureus* cells were grown at 37°C with aeration in CYGP 03GL broth (39) or tryptic soy broth supplemented with antibiotics as indicated. Luria-Bertani (LB) was used for cultivating *Escherichia*

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* RN4220, 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* (*rat*) 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 Φ11 and 80α phage lysates of ALC2530 to infect strains COL, Newman, FDA486, RN6911 (*agr* mutant), and ALC3043 (*sarT* mutant) (Table 1). For the construction of the *sarT agr* double mutant (ALC3188), a Φ11 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 [OD_{650}] = 1.2) phase. The cells were harvested and processed with a Trizol isolation kit (Gibco BRL, Gaithersburg, Md.) in combination with 0.1-mm sirconia-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 Hybond N^+ membranes (Amersham, Arlington Heights, Ill.) was performed with the Turboblotter alkaline transfer system (Schleicher & Schuell, Keene, N.H.). For detection of specific transcripts (RNAIII, *sarA*, *saeRS*, *sarS*, *sarT*, *spa*, *hla*, and *mgrA*), gel-purified DNA probes were radiolabeled with [α-^{32}P]dCTP by the random-primed DNA labeling method (Roche Diagnostics GmbH) and hybridized under aqueous-phase conditions at 65°C (6). The blots were subsequently washed and autoradiographed as described (11).

Transcriptional fusion studies of different promoters linked to the *gfp$_{uvr}$* reporter gene. To confirm the effect of the *mgrA* mutation on promoter activities of other regulators (RNAIII and *sarS)* 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 *gfp$_{uvr}$* gene) upstream of the *gfp$_{uvr}$* 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 (OD_{650}) and fluorescence for 10 h in a FL600 fluorescence spectrophotometer (BioTek Instrument, Winooski, Vt.). Promoter activities were plotted as mean fluorescence/OD_{650} ratio to minimize variations due to cell density, using the average values from triplicate readings.

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 235-bp *hla* promoter fragment (nucleotides 1 to 80 plus 155 bp upstream of the start codon) (13) were end-labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. Similarly, a 264-bp *sarS* 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*, *sae*, 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.* Northern blot analysis revealed that the expression of *hla* was reduced in the *mgrA* mutant compared with the parent (RN6390), while the *spa* transcript level was increased (Fig. 1A). As *sarA*, *sae*, and *agr* were known to modulate *hla* and *spa* transcription, we analyzed transcript levels of these regulatory loci in the *mgrA* mutant of strain RN6390. Interestingly, the transcript levels of *sarA* and *saeRS*, two known global regulators of virulence, were unchanged (Fig. 1B). In contrast, there was a significant decrease in the mRNA level of RNAIII of *agr* in the mutant compared with the parent, while the *sarS* level was increased (Fig. 1C).

To confirm the effects of the *mgrA* mutation on RNAIII, *sarS*, *hla*, and *spa* transcription, we transformed strain RN6390 and its isogenic *mgrA* mutant with shuttle plasmids carrying the *gfp$_{uvr}$* reporter gene driven by the RNAIII, *sarS*, *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).

SarT is a known *hla* repressor that is normally repressed by *agr* (49). Despite the finding that *hla* expression was downregulated in the *mgrA* mutant, we were not able to detect any significant *sarT* transcript in the parent strain as well as in the *mgrA* mutant. Promoter fusion studies also verified that *sarT* was weakly expressed in the wild type and the *mgrA* mutant (data not shown). Thus, SarT either does not play a major role in the *mgrA*-mediated regulation of *hla* expression, or the low level of *sarT* detection precludes us from discriminating the contribution of *sarT* to *hla* regulation by *mgrA* (see below).

To ensure that these observations are not unique to the *mgrA* mutant 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

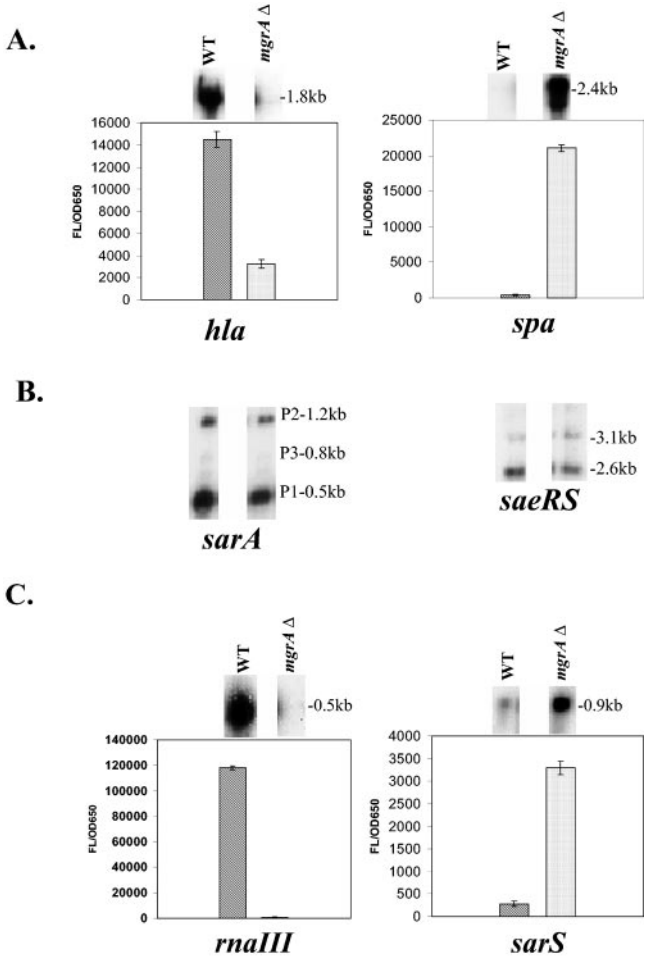

FIG. 1. Effect of the *mgrA* mutation on the expression of genes involved in virulence. A. Northern blot analysis of *hla* and *spa* transcripts in RN6390 and isogenic *mgrA* mutant ALC2530. The open reading frame of each gene was used as a probe for the Northern blots. RNA was harvested from cells grown to an OD_{650} 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/OD_{650} 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; ▨, *mgrA* mutant ALC2530. B. Northern blot analysis of *sarA* and *saeRS* 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 OD_{650} 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 OD_{650} 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/OD_{650} 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; ▨, mgrA mutant ALC2530).

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*.

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 pI524, 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

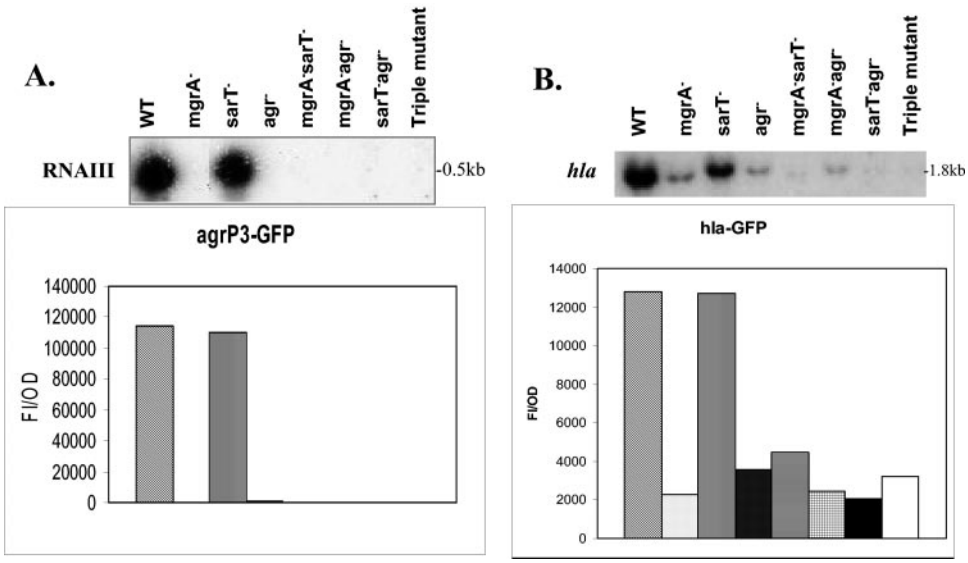

FIG. 2. Transcription of *agr* RNAIII and *hla* in RN6390 and isogenic mutants. A. Northern blot analysis of *agr* RNAIII transcripts in RN6390 and isogenic mutants. The probe for RNAIII was a 920-bp fragment containing the *hld* gene. RNA was harvested from cells grown to an OD_{650} of ≈1.2, representing late exponential phase. The expression of GFP driven by the *agr* RNAIII (P3) 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. B. Northern blot analysis of the *hla* transcript in RN6390 and isogenic mutants. The probe for *hla* was an 800-bp fragment encompassing the open reading frame. RNA was harvested from cells grown to an OD_{650} of ≈1.2, representing late exponential phase. The expression of GFP driven by the *hla* 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.

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

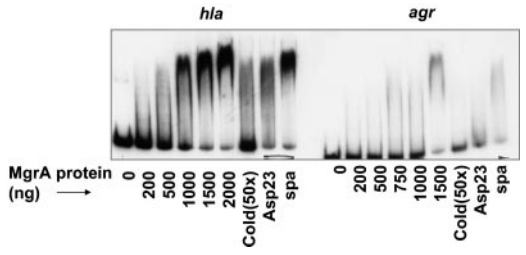

FIG. 3. Gel shift assays of purified MgrA with *hla* and *agr* promoter fragments. MgrA protein in increasing concentrations was incubated with end-labeled *hla* or *agr* promoter fragments. In competition assays, MgrA protein (1,500 ng) was incubated with the promoter fragment in the presence of a 50-fold excess of unlabeled specific competitor (*hla* or *agr*) (lane 7) or nonspecific competitors (≈300-bp *asp23* promoter in lane 8 and ≈160-bp *spa* promoter fragment in lane 9).

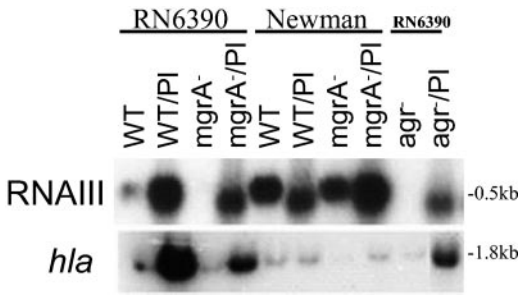

FIG. 4. Transcription of *hla* by providing RNAIII in *trans.* Northern blot analysis of *agr* RNAIII and *hla* transcripts in strains with and without plasmid RN6735. (Transformants are indicated as strain/Pl.) RNA was harvested from cells grown to an OD_{650} of ≈1.2, representing late exponential phase.

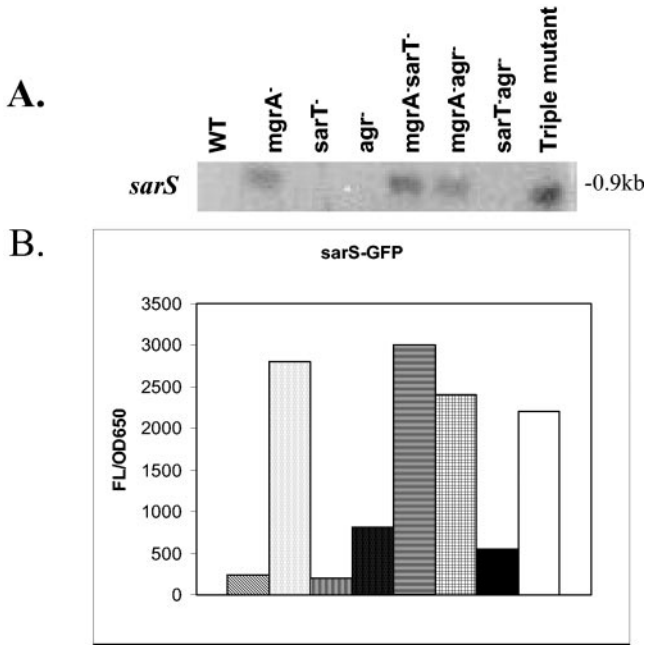

FIG. 5. Transcription of *sarS* in RN6390 and isogenic mutants. A. Northern blot analysis of the *sarS* transcript in RN6390 and isogenic mutants. The open reading frame of *sarS* was used as a probe. RNA was harvested from cells grown to an OD_{650} of ≈1.2, representing late exponential phase. B. The 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.

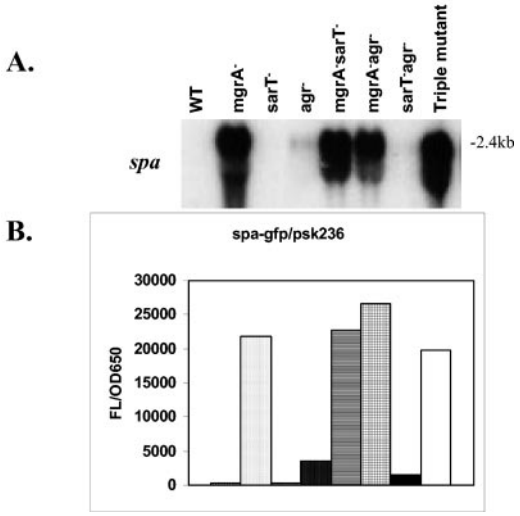

FIG. 6. Transcription of *spa* in RN6390 and isogenic mutants. A. Northern blot analysis of the *spa* transcript in RN6390 and isogenic mutants. The open reading frame of *spa* was used as a probe. RNA was harvested from cells grown to an OD_{650} of ≈1.2, representing late exponential phase. B. The expression of GFP driven by the *spa* 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 downregulation 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 ηg of MgrA was required to retard the mobility of the *sarS* promoter (Fig. 7). At 2,000 ηg, 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.

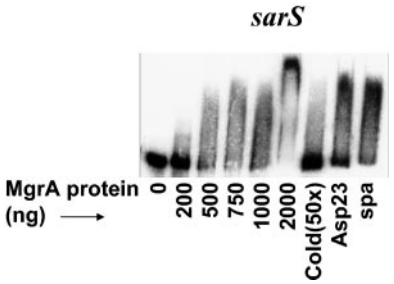

FIG. 7. Gel shift assays of purified MgrA with *sarS* promoter fragment. MgrA protein in increasing concentrations was incubated with the end-labeled *sarS* promoter fragment. In competition assays, MgrA protein (1,000 ng) was incubated with end-labeled promoter in the presence of a 50-fold excess of 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).

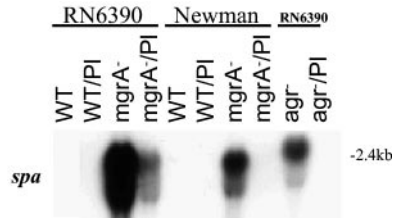

FIG. 8. Transcription of *spa* by providing RNAIII in *trans.* Northern blot analysis of the *spa* transcript in strains with and without plasmid RN6735. (Transformants are indicated as strain/Pl.) The open reading frame of the *spa* gene was used as a probe. RNA was harvested from cells grown to an OD_{650} of ≈1.2, representing late exponential phase.

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 major players within this network appear to be the two-component systems (e.g., *agrAC*, *saeRS*, *arlRS*, and *srrAB*) and members of the SarA protein family (8, 12, 16, 18, 26, 44). Based on structure and sequence alignment, the SarA protein family can be divided into three subfamilies: the single-domain proteins (e.g., SarA and SarR), the double-domain proteins (e.g., SarS and SarU), and the MarR homologs (e.g., MgrA).

In previous studies (11, 32), we have shown that members of the single-domain (SarR on *sarA* expression) and double-domain (SarS on *spa* expression) proteins participate in the modulation of virulence determinants. The first member of the third SarA subfamily, MgrA, was originally identified as an important regulator of autolytic activity in *S. aureus* (21, 35). We have now characterized *mgrA* further with regard to the expression of virulence determinants and conclude that all three SarA subfamilies likely participate in the virulence regulatory network.

We have examined in this study four prototypic *S. aureus* strains, including RN6390, Newman, FDA486, and COL. Inactivation of *mgrA* in all these strains has led to downregulation of RNAIII of *agr*, decreased expression of *hla*, and upregulation of *sarS* and *spa* (Fig. 1 and 2). Phenotypically, decreased expression of *hla* and upregulation of *spa* in an *mgrA* mutant is consistent with an *agr* mutant phenotype. However, the effect of *mgrA* on intermediate regulatory genes such as *sarS* and *sarT* downstream of *agr* as well as target genes (direct versus indirect effect) has not been previously defined. In addition, we have also found that the transcription of *sarA*, an important regulatory locus that partially controls *agr*, is not affected in the *mgrA* mutant, suggesting that *mgrA* likely regulates *hla* and *spa* in a SarA-independent manner.

Regulation of *hla* expression in *S. aureus* is not mediated by a single locus but is multifaceted. In particular, regulators such as *sarA*, *agr*, and *sae* have been shown to upmodulate *hla* expression both directly and indirectly (5, 13, 17, 22, 42, 51), while *sarT* downregulates it (49). Similarly, *spa* expression is repressed by *sarA* and *agr* and promoted by *sarT* and *sarS* (10, 48, 52,). Added to this list now is *mgrA*, which is part of the regulatory cascade that controls *agr* and *sarS* to modulate *hla* and *spa* expression.

Analysis of the double *sarT mgrA* 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 Tn*917* transposon screen, searching for mutants that had altered capsular production in *cap8*$^+$ 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

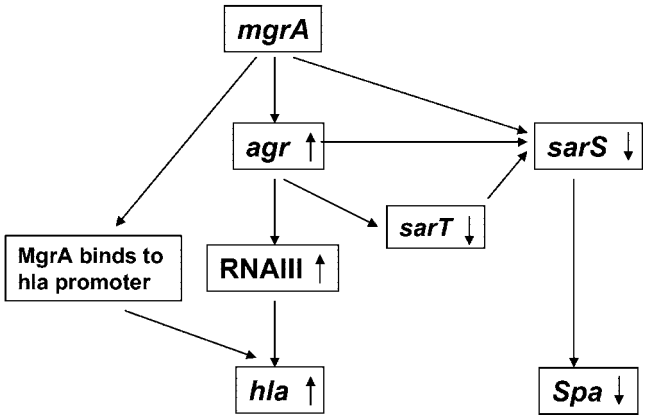

FIG. 9. Model for MgrA-mediated bimodal regulation of virulence genes in *S. aureus*. MgrA upregulates expression of RNAIII. RNAIII activates *hla* expression and downregulates *sarS*, leading to repression of *spa*. Besides the *agr*-dependent pathway, MgrA binds directly to the *hla* promoter to augment 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.*

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