

MgrA Represses Biofilm Formation in *Staphylococcus aureus*[▽]

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MgrA is a pleiotropic regulator that controls autolysis, virulence, and efflux pump activity in *Staphylococcus aureus*. We recently found that *mgrA* mutants of strains RN6390, SH1000, and MW2 also displayed enhanced biofilm formation compared with their respective parents. The biofilms formed by *mgrA* mutants of RN6390 and MW2 are independent of *sigB* and *ica* loci, two genetic elements that have been previously associated with biofilm formation in *S. aureus*. Biofilms formed by *mgrA* mutants are dependent on the expression of surface proteins mediated by the sortase gene *srtA*. Extracellular DNA was also a crucial component of the early biofilm of *mgrA* mutants. Genetic analysis indicated that biofilm formation in *mgrA* mutants is mediated in part by *agr* RNAIII, a genetic locus regulated by *mgrA*. Additionally, SarA is important to biofilm formation in *mgrA* mutants since the double *sarA mgrA* mutants failed to form biofilms compared to single *mgrA* mutants of RN6390 and MW2. However, the SarA-mediated effect is independent of *agr* and proteases such as V8 protease and aureolysin. Collectively, our data showed MgrA to be a repressor of biofilm formation, and biofilms formed by *mgrA* mutants have features that are distinct from other reported biofilm types in *S. aureus*.

Staphylococcus aureus is a major human pathogen that is a common cause of community- and hospital-acquired infections (22). These infections have been problematic due in part to a dramatic increase in antibiotic resistance (e.g., methicillin-resistant *S. aureus* [MRSA]). The problem with antibiotic resistance can be traced in part to *S. aureus*'s ability to produce biofilms (14, 26). Biofilms constitute a protected environment of growth that enables the bacteria to proliferate by restricting antibiotic access and shielding the bacterial pathogen from host immune defenses (11). The matrices of bacterial biofilms generally consist of polysaccharides, surface proteins, and, in some cases, extracellular DNA (13, 35). However, the expression and specific composition of individual biofilms can differ among bacterial species and are dependent on the metabolic state and environmental conditions (13).

Bacterial biofilms are purported to be important in chronic infections (13). Despite their clinical significance, the expression and regulation of *S. aureus* biofilms remain poorly defined. Several *S. aureus* genes have been shown to be crucial to biofilm formation, including *ica*, *arlRS*, *agr*, and *sarA* (25, 38, 39, 43). The extracellular polysaccharide adhesin PIA/PNAG, (polysaccharide intracellular adhesion/poly-*N*-acetylglucosamine) encoded by the *icaADBC* locus (25), has been shown to be responsible for some of the biofilm-positive phenotypes (adhesion, microaggregation, and macroaggregation) in *S. aureus* and, to a greater extent, in *Staphylococcus epidermidis* (43). However, recent studies have shown that *S. aureus* can produce an alternative *ica*-independent biofilm (9, 10), especially in the absence of the two-component regulatory system *arlRS*, thus indicating that PIA/PNAG is not an essential component of all biofilm matrices in *S. aureus* (38).

Another two-component regulatory system linked to biofilm

formation is the accessory gene regulator (*agr*), which entails a complex quorum-sensing scheme. The *agr* locus consists of two divergent transcripts, RNAII and RNAIII, which carry *agr*DBCA and *hld*, respectively (31). RNAIII is the *agr* effector molecule that enhances the expression of exoprotein genes while downregulating genes encoding surface proteins. The inactivation of *agr* leads to an enhanced biofilm phenotype, in part due to the increased expression of surface adhesive proteins (32). The *agr* locus is also activated in part by SarA, a DNA binding protein that belongs to a family of transcription factors in *S. aureus* called the SarA protein family (5). The *sarA* locus, encoding the 14.5-kDa SarA protein, is required for optimal *ica* expression and also for the controlled expression of Bap, a surface protein essential for biofilm formation in bovine *S. aureus* isolates (39). Additionally, a recent study divulged that *cidA*, encoding a putative holin molecule, may mediate DNA release by triggering cell lysis during biofilm formation in *S. aureus* (35). However, the exact role of DNA release in the different stages of biofilm formation has not been defined.

In this study, we demonstrate that the inactivation of *mgrA*, a negative regulator of autolysin genes, enhances biofilm formation via a *sigB*- and *ica*-independent manner. This effect is likely mediated by surface proteins regulated by *srtA*. Additionally, extracellular DNA released within the biofilm is also an essential matrix component in early biofilms formed by *mgrA* mutants. Genetic analyses have implicated *agr* and *sarA* as playing important roles in biofilm formation in *mgrA* mutants.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Phages ϕ 11 and 80 α were used to transduce *S. aureus* strains. *S. aureus* cells were grown at 37°C with aeration in CYGP or 03GL broth (30) or tryptic soy broth (TSB) supplemented with antibiotics when necessary. Luria-Bertani broth was used for cultivating *Escherichia coli*. Antibiotics were used at the following concentrations: for *S. aureus*, erythromycin at 5 μ g/ml, tetracycline at 3 μ g/ml, chloramphenicol at 10 μ g/ml, and kanamycin at 50 μ g/ml and, for *E. coli*, ampicillin at 50 μ g/ml.

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TABLE 1. Strains and plasmids used in this study

| Strain or plasmid | Description | Reference |
|-------------------|---|------------|
| Strains | | |
| <i>S. aureus</i> | | |
| RN4220 | Mutant strain of 8325-4 that accepts foreign DNA | 30 |
| RN6911 | <i>agr</i> mutant of RN6390 with complete deletion of RNAPII and RNAPIII (Δ <i>agr::tetM</i>) | 31 |
| SA113 | Common laboratory isolate | 10 |
| RN6390 | <i>agr</i> ⁺ laboratory strain related to 8325-4 but with a defective <i>rsbU</i> | 33 |
| SH1000 | Functional <i>rsbU</i> derivative of 8325-4 | 15 |
| MW2 | Community-associated methicillin-resistant strain | 2 |
| ALC4006 | RN6390 Δ <i>mgrA</i> | This study |
| ALC6226 | RN6390 Δ <i>mgrA</i> pCL84- <i>mgrA</i> | 19,28 |
| ALC6214 | RN6390 Δ <i>mgrA</i> Δ <i>srtA</i> | This study |
| ALC2057 | RN6390 Δ <i>sarA::kan</i> | 16 |
| ALC5408 | RN6390 Δ <i>mgrA</i> Δ <i>sarA::kan</i> | This study |
| ALC6216 | RN6390 Δ <i>mgrA</i> Δ <i>sarA::kan</i> Δ <i>aur::ermB</i> | This study |
| ALC6215 | RN6390 Δ <i>mgrA</i> Δ <i>sarA::kan</i> Δ <i>sspA::ermB</i> | This study |
| ALC5413 | SH1000 Δ <i>mgrA</i> | This study |
| ALC6225 | SH1000 Δ <i>mgrA</i> pCL84- <i>mgrA</i> | This study |
| ALC2892 | SH1000 Δ <i>sarA::kan</i> | This study |
| ALC5414 | SH1000 Δ <i>mgrA</i> Δ <i>sarA::kan</i> | This study |
| ALC6228 | SH1000 Δ <i>mgrA</i> Δ <i>sarA::kan</i> Δ <i>aur::ermB</i> | This study |
| ALC6229 | SH1000 Δ <i>mgrA</i> Δ <i>sarA::kan</i> Δ <i>sspA::ermB</i> | This study |
| ALC5419 | MW2 Δ <i>mgrA</i> | This study |
| ALC6230 | MW2 Δ <i>mgrA</i> pCL84- <i>mgrA</i> | This study |
| ALC6218 | MW2 Δ <i>mgrA</i> Δ <i>srtA</i> | This study |
| ALC6259 | MW2 Δ <i>agr</i> RNAPII/III:: <i>tetM</i> | This study |
| ALC5415 | MW2 Δ <i>sarA::kan</i> | This study |
| ALC5420 | MW2 Δ <i>mgrA</i> Δ <i>sarA::kan</i> | This study |
| <i>E. coli</i> | | |
| XL1-Blue | Host strain for cloning | |
| Plasmids | | |
| pALC2464 | pCL84 with the complete <i>mgrA</i> gene (pCL84- <i>mgrA</i>) | This study |
| pMAD | <i>E. coli</i> - <i>S. aureus</i> shuttle vector with a thermosensitive origin of replication for gram-positive bacteria and the <i>bgab</i> gene encoding β -galactosidase | 1 |
| pALC3371 | pMAD plasmid containing the mutant allele for deletion of <i>mgrA</i> (pMAD:: Δ <i>mgrA</i>) | This study |
| pALC6199 | pMAD plasmid containing the mutant allele for deletion of the <i>srtA</i> gene (pMAD:: Δ <i>srtA</i>) | This study |
| pRN6735 | Contains promoterless RNAPIII under the control of the <i>blaZ</i> promoter | 31 |
| pALC1484 | Derivate of pSK236, containing the recombinant <i>gfp_{uvr}</i> gene | 20 |
| pALC6211 | pALC1484 with a 194-bp promoter fragment of <i>ica</i> fused with the <i>gfp_{uvr}</i> reporter gene at the KpnI/EcoRI sites | This study |
| pALC1743 | pALC1484 with the 229-bp <i>agr</i> RNAPIII promoter fragment fused with the <i>gfp_{uvr}</i> reporter gene at the EcoRI/XbaI sites | 17 |

Genetic manipulations in *E. coli* and *S. aureus*. Construction of the recombinant plasmids was performed in *E. coli* XL1-Blue with standard molecular biology and recombinant DNA techniques as described by Maniatis et al. (27). Restriction enzymes were purchased from New England Biolabs and used according to the manufacturer's instructions. Primers were obtained from Operon Technology. *Staphylococcus aureus* strain RN4220, a restriction-deficient derivative of strain 8325-4, was used as the initial recipient of the plasmid constructs by electroporation (36).

The *sarA*, *aur*, and *sspA* mutants of *S. aureus* were constructed by transducing the *sarA::kan*, *aur::ermB*, and *sspA::ermB* mutations from 8325-4 to recipient strains, using phage ϕ 11.

To construct the *mgrA* mutant, we amplified by PCR two fragments that flanked the left and right sides of the gene sequence targeted for deletion. The two resulting PCR fragments have a 16-base complementary region to facilitate annealing followed by a second round of PCR amplification with outside primers to obtain a single fragment. The fusion product was purified, digested with SmaI, and ligated into shuttle vector pMAD. A similar strategy was also used to construct deletion mutants of *srtA* and *agr* in *S. aureus* strains. The resulting recombinant pMAD plasmids (Table 1) were transformed first into *S. aureus* RN4220 and then into the target strain by electroporation. The allelic exchange in the absence of the selection marker was performed as previously described (41). Briefly, the plasmid pMAD, containing a temperature-sensitive *S. aureus*

origin of replication, an erythromycin resistance cassette, and a β -galactosidase gene (*bgab*), was integrated into the host chromosome at the nonpermissive temperature (44°C), resulting in a light-blue erythromycin-resistant colony on X-Gal (5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid; 150 μ g/ml) plates. One to five light-blue colonies were picked onto 10 ml of TSB and incubated overnight at 30°C without any antibiotic. Tenfold serial dilutions of this culture were plated onto Trypticase soy agar plates containing X-Gal. White and erythromycin-sensitive colonies, which no longer contained the pMAD plasmid, were then selected as plausible allelic replacement mutants, which were confirmed by PCR and Southern blots with the appropriate probes.

Transcriptional fusion studies of the *ica* and the RNAPIII promoters linked to the *gfp_{uvr}* reporter gene. To confirm the effect of the Δ *mgrA* mutation on the promoter activity of *ica* and RNAPIII, we used the shuttle plasmids pALC6211 and pALC1743 containing the *icaA* and the *agr* RNAPIII promoters, respectively, driving the *gfp_{uvr}* reporter gene (Table 1). In brief, the 164-bp region between *icaR* and *icaA*, representing the *ica* promoter driving *icaADBC* expression (19), was cloned upstream of the *gfp* reporter in shuttle plasmid pALC1484 to yield pALC6211. Similarly, the *agr* RNAPIII promoter was also cloned upstream of pALC1484 to yield pALC1743 (17). The plasmids pALC6211 and pALC1743 were transformed into RN4220, purified from RN4220 transformants, and then electroporated into RN6390 and/or MW2 and its isogenic Δ *mgrA* mutants. To detect promoter activity, overnight cultures of *S. aureus* strains harboring either

pALC6211 or pALC1743 were diluted 1:50 in TSB containing chloramphenicol (10 $\mu\text{g}/\text{ml}$) and grown at 37°C with shaking. Aliquots (200 μl) were transferred in triplicate every 30 min to microtiter plates to assay for cell density (optical density at 595 nm [OD₅₉₅]) and fluorescence for a 6- to 10-h period in an FL600 microplate fluorescence reader (BioTek Instruments, Winooski, VT). Promoter activation was plotted as the mean fluorescence/OD₅₉₅ ratio to minimize variations from different cell densities, using the average values from triplicate readings.

Biofilm formation. *S. aureus* strains were grown overnight and diluted 1:40 in TSB supplemented with 0.25% glucose (TSB-glucose). Two hundred microliters each of this cell suspension was used to inoculate sterile 96-well polystyrene microtiter plates (Costar) in triplicate. After 16 h of incubation at 37°C, the wells were washed three times with sterile water, stained with 0.1% crystal violet for 1 min, and washed again three times with water. Photographs of the inverted plate were taken with a scanner. To quantitate the biofilms, the crystal violet stain was solubilized with 30% glacial acetic acid for 15 min. The relative biofilm formation was determined by reading the OD₅₆₂, using a Bio-Tek microplate reader (Bio-Tek FL600).

Colony morphology on CRA. Colony morphology on Congo red agar (CRA) was determined as previously described (7). Briefly, a biofilm-positive strain is linked with rough colonies, while a biofilm-negative strain is associated with smooth colonies.

Biofilm detachment assays. Biofilms were grown for 16 h in the wells of 96-well microtiter plates as described above. The biofilms were washed with water and then treated for 2 h at 37°C with 100 μg of proteinase K per ml (Sigma) in 20 mM Tris buffer (pH 7.5) or 0.14 units of DNase I in 20 mM Tris buffer (pH 7.5). After treatment, the biofilms were washed with water, stained with crystal violet, and quantitated as described above. Biofilm detachment assays were performed three times with similar results. In some assays, proteinase K and DNase I were added to the TSB-glucose culture medium in the microtiter wells before formation of the biofilm.

PIA/PNAG detection. PIA/PNAG production in *S. aureus* strains was detected as described by Cramton et al. (8). Briefly, cells were grown in TSB-glucose, and the same number of cells from each culture was resuspended in 50 μl of 0.5 M EDTA (pH 8.0), incubated for 5 min at 100°C, and centrifuged. Forty microliters of the supernatant was incubated with 10 μl of proteinase K (20 mg/ml; Sigma) for 30 min at 37°C. One microliter was then spotted on a nitrocellulose filter using a Bio-Dot microfiltration apparatus (Bio-Rad), blocked overnight with 5% skimmed milk in phosphate-buffered saline with 0.1% Tween 20, and incubated for 2 h with rabbit anti-*S. aureus* PIA/PNAG antibody diluted 1:5,000 (4). Bound antibodies were detected with a peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody (Jackson ImmunoResearch Laboratories) diluted 1:10,000 and developed with the GE Health Care ECL kit.

RESULTS

***mgrA* represses biofilm formation in *S. aureus*.** In previous studies, we have shown that MgrA is a negative regulator of autolysis in *S. aureus* (16). Specifically, the *mgrA* mutant has a mild growth defect during the late postexponential phase and exhibited increased sensitivity to lysis upon exposure to Triton X-100 and penicillin (16). Since an enhanced autolytic phenotype has been associated with biofilm formation (12), we wanted to determine if an *mgrA* mutant also exhibited an enhanced biofilm phenotype in various *S. aureus* strains.

We initially chose to study RN6390, a methicillin-sensitive *S. aureus* (MSSA) laboratory isolate with an impaired *rsbU* gene, and SH1000, a derivative of 8325-4 in which the *rsbU* mutation has been repaired (15). As shown in Fig. 1, both *mgrA* mutants, irrespective of their *rsbU* status, were able to produce more biofilm on polystyrene plates than their respective parental controls. To verify that this effect is not limited to MSSA strains, we constructed an *mgrA* deletion mutant of MW2, a community-acquired MRSA strain, which also displayed enhanced biofilm formation with an *mgrA* mutation (Fig. 1). The enhanced biofilm phenotype correlated with the presence of rough *mgrA* mutant colonies on Congo red agar, while the parental strains displayed a smooth-colony phenotype (Fig. 1,

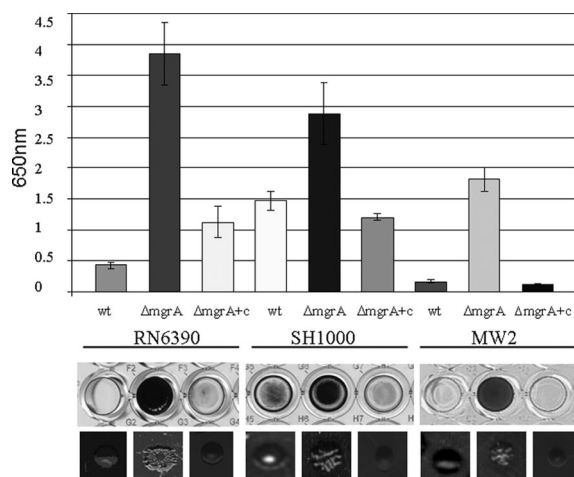


FIG. 1. Biofilm formation by *mgrA* mutants. Top panel, biofilm formation of the *mgrA* mutants, wild types (wt), and their respective complemented strains (c) was quantified by solubilizing the crystal violet stain in 30% glacial acetic acid and measuring the absorbance at 562 nm. As assessed by the Student *t* test, the significance of the data was as follows: the *mgrA* mutant of RN6300 versus the parent and complement, $P < 0.0004$; the *mgrA* mutant of SH1000 versus the parent and complement, $P < 0.03$; the *mgrA* mutant of MW2 versus the parent and mutant, $P < 0.0001$. Bottom panel, biofilm formation of *mgrA* mutants and their complemented strains on microtiter plates grown in TSB supplemented with glucose (0.25%) at 37°C over 16 h. A characterization of the colony morphology of different *mgrA* mutants and their respective complemented strains on CRA plates is presented below.

bottom panel). The biofilm phenotype and colonial morphology on CRA in *mgrA* mutants were restored to those of the respective parents upon complementation (Fig. 1). Collectively, these findings indicated that biofilm formation in *mgrA* mutants is *rsbU* independent and can occur in both MSSA and MRSA strains.

The *ica* locus is not required for biofilm formation in *mgrA* mutants. Previous studies have shown that *S. aureus* can develop PIA-dependent and PIA-independent biofilms (8, 38, 41). To determine if the biofilm in an *mgrA* mutant is reliant on PIA, a dot blot analysis of cell surface extracts from overnight cultures of RN6390 and MW2 and their isogenic *mgrA* mutants was performed on nitrocellulose membranes as described in Materials and Methods. PIA (PNAG), encoded by the *icaADBC* locus, was detected with anti-PIA polyclonal antibody (a gift of Gerald Pier). As shown in Fig. 2A, the *mgrA* mutants of RN6390 and MW2, with an enhanced biofilm phenotype, displayed a small reduction in PIA expression compared with the isogenic parents, while the positive-control *S. aureus* strain SA113 expressed PIA at a high level. To verify the dot blot analysis, we conducted transcriptional fusion assays of an *ica* promoter driving the *gfp* reporter gene in pALC1484 in strains RN6390 and MW2 and their isogenic *mgrA* mutants. This 164-bp *ica* promoter fragment which spans the entire region between the *icaR* and *icaA* open reading frames has previously been shown by another research group to be active in *ica* transcription (19). The fluorescence attributable to the *ica* promoter activity was ranging from ~6,000 to 8,000 fluorescence units/OD₅₉₅ in these strains and did not differ significantly between the *mgrA* mutants and their respective parents

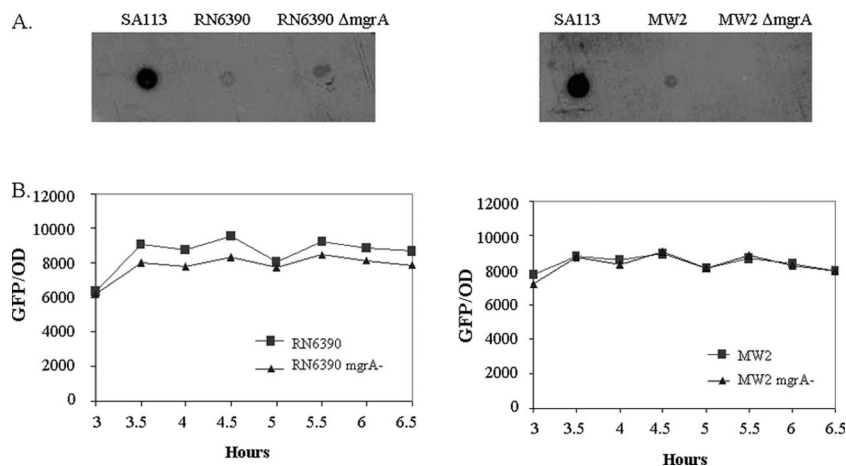


FIG. 2. (A) Dot blot analysis of PIA/PNAG accumulation in *mgrA* mutants and wild-type strains. Cell surface extracts from overnight cultures of *S. aureus* wild-type strains RN6390 and MW2, their *mgrA* mutants, and the positive control SA113, treated as described in Materials and Methods, were placed onto nitrocellulose membranes. PIA/PNAG production was detected with an anti-PIA/PNAG polyclonal antibody. (B) Quantification of the activity of the 164-bp *ica* promoter (19) in the *mgrA* mutants and their corresponding wild types by transcriptional fusion studies. The expression of green fluorescent protein driven by the *ica* promoter was measured as a fluorescence/OD₅₉₅ ratio using average values of triplicate readings. This experiment was repeated at least three times, with one representative experiment shown.

(Fig. 2B). Notably, the baseline fluorescence for this assay with the shuttle plasmid pALC1484 alone without the *ica* promoter was ~200 fluorescence units, thus indicating that the *ica* promoter was active in both parental strains and their isogenic *mgrA* mutants.

Biofilm formation in an *mgrA* mutant is dependent in part on *srtA*. Surface proteins often play an important role in early adhesion and intercellular aggregation in the formation of biofilms (24). To examine the role of surface protein adhesins in the biofilms formed by *mgrA* mutants, we explored whether the sortase gene (*srtA*), which serves to anchor many cell wall proteins covalently to the staphylococcal cell wall (29), would affect biofilm formation in *mgrA* mutants. Accordingly, we deleted *srtA* in the *mgrA* mutants and found that the double mutants had significantly reduced biofilm formation compared with the respective single *mgrA* mutants while the parental strains RN6390 and MW2 were poor biofilm formers in this assay (Fig. 3). We thus deduced that surface proteins anchored

by sortase A probably play a major role in biofilm formation in *mgrA* mutants, presumably due to the increased expression of surface adhesins and/or cellular aggregation.

The *mgrA* mutant biofilm is composed of proteins and DNA.

The composition of *S. aureus* biofilms has been shown to vary with metabolic state, growth conditions, and genetic mutations (24). To ascertain if surface proteins are indeed important constituents of biofilms in *mgrA* mutants, preformed biofilms of *mgrA* mutants of RN6390 and MW2 after overnight growth in TSB-glucose were treated for 2 h with proteinase K (100 μ g/ml), and the residual attached bacteria in the biofilm were then stained with crystal violet. As displayed in Fig. 4, treatment of the preformed biofilms of *mgrA* mutants with proteinase K eliminated the biofilms to the level of the respective parents. Similarly, the addition of proteinase K to TSB-glucose medium prior to overnight growth also abolished the formation of biofilms of *mgrA* mutants. Given that proteinase K cannot lyse *S. aureus* (data

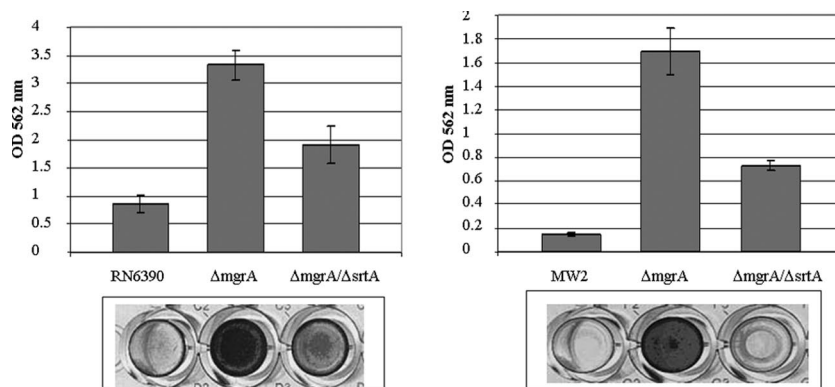


FIG. 3. Biofilm formation phenotype of the double $\Delta mgrA \Delta srtA$ mutants of RN6390 and MW2. Significant differences in biofilm formation capacity were found between the *mgrA* mutant and the $\Delta mgrA \Delta srtA$ double mutant. The significance data obtained with the Student *t* test were as follows: the *mgrA* mutant versus RN6390, $P < 0.0001$; the *mgrA srtA* mutant versus the *mgrA* mutant of RN6390, $P < 0.0012$; the *mgrA* mutant versus MW2, $P < 0.0002$; and the *mgrA srtA* mutant versus the *mgrA* mutant of MW2, $P < 0.001$. The microtiter plate results are shown below.

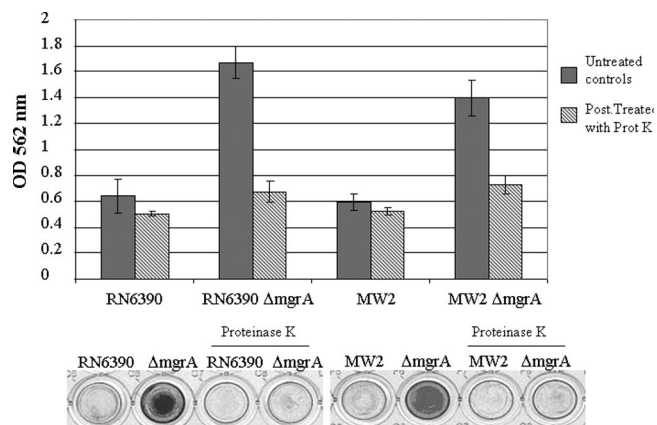


FIG. 4. Detachment of preformed biofilms by proteinase K. The biofilms of RN6390 and MW2 and their isogenic $\Delta mgrA$ mutant strains grown in TSB-glucose for 16 h at 37°C were treated for 2 h at 37°C with 100 μ g of proteinase K/ml (striped bars). The bacteria that remained attached in the microtiter plate were stained with crystal violet. The dye was dissolved with 30% glacial acetic acid (200 μ l per well) and measured by OD₅₆₂. The untreated controls are represented by filled bars. The microtiter views are shown below.

not shown), it is likely that surface proteins that mediate bacterial attachment or aggregation are eliminated as a result of proteinase K treatment.

Recent studies by Rice and colleagues (35) have also implicated released DNA from lysed cells in biofilm formation. To determine if DNA is an essential component of the early biofilms of $mgrA$ mutants, DNase I was added to the TSB-glucose medium containing $mgrA$ mutants of RN6390 or MW2. After overnight growth, the formation of a biofilm was quantified by measuring the OD₅₆₂ of the released crystal violet stain. As shown in Fig. 5, the overnight treatment of cultures of $mgrA$ mutants with DNase I (0.7 units/ml) has led to the eradication of biofilms compared with nontreated controls. Interestingly, the addition of DNase I at concentrations ranging from 1 to 64 units/ml to preformed biofilms of $mgrA$ mutants did not abolish the biofilm (data not shown). This finding indicated that extracellular DNA is an important component of the early biofilms of $mgrA$ mutants but not in preformed biofilms. However, with the concentrations of DNase I used in our study, we could not rule out a lack of access to DNA by DNase I in established biofilms.

Biofilm formation of the $mgrA$ mutant is mediated by agr . As agr represses the expression of surface proteins and its expression is reduced in $mgrA$ mutants (17), we hypothesized that $mgrA$ mutants may enhance biofilm formation by downregulating agr . The agr locus is composed of two divergent transcripts, RNAII and RNAPIII, bearing $agrDBCA$ and hld , respectively. Accordingly, we first examined biofilm formation in agr mutants lacking both RNAII and RNAPIII in strains RN6390 and MW2 (Fig. 6A). As expected, a deletion in the agr operon including RNAII and RNAPIII resulted in augmented biofilm formation in both RN6390 and MW2, thus emphasizing the importance of agr in repressing biofilm development. The additional deletion of $mgrA$ in the agr mutants did not significantly alter biofilm formation in comparison to single agr mu-

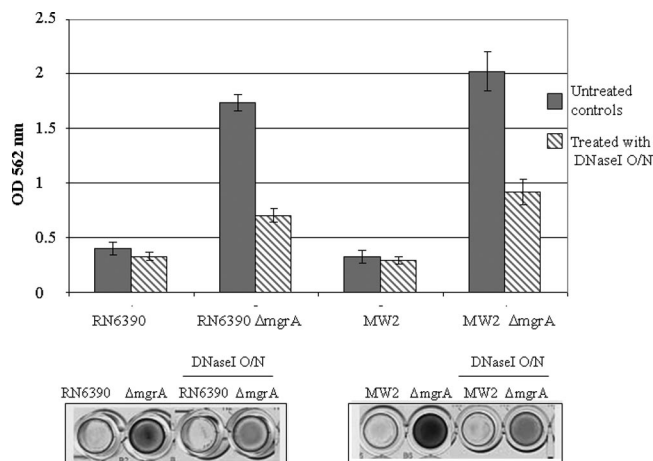


FIG. 5. Disruption of the early biofilm formation of $mgrA$ mutants treated with DNase I. The biofilms of RN6390 and MW2 and their corresponding $\Delta mgrA$ mutant strains grown in 200 μ l of TSB-glucose in microtiter wells for 16 h at 37°C in the presence of DNase I (0.7 units per ml) are represented by the striped bars. Biofilm formation was quantified by washing the biofilms three times with water, staining with crystal violet, and measuring the OD₅₆₂. The filled bars are the untreated controls. The corresponding views of the microtiter plates are shown below.

tants, consistent with the notion that $mgrA$ and agr may modulate a similar pathway in biofilm formation.

To provide credence that biofilm formation in $mgrA$ mutants is attributable to reduced agr expression, we provided RNAIII expression in *trans* in $mgrA$ mutants of RN6390 and MW2 with the plasmid pRN6735, which expressed RNAIII under the control of a *blaZ* promoter (42). Remarkably, the provision of RNAIII in *trans* significantly reduced biofilm formation in $mgrA$ mutants but not to the level of the respective parents (Fig. 6B). Collectively, these results indicated that enhanced biofilm formation in $mgrA$ mutants is in large part due to the reduced expression of RNAIII of agr as well as to agr -independent but $mgrA$ -mediated factors.

To verify further that $mgrA$ indeed regulates agr in our strain, we introduced the shuttle plasmid pALC1743, a derivative of pSK236 containing the RNAIII promoter driving gfp_{uvr} , into MW2 and its isogenic $mgrA$ mutant. Fluorescence activities were then measured in these two isogenic strains over a 10-h period and then overnight. As shown in Fig. 6C, fluorescence attributable to agr RNAIII promoter activity was highest during the stationary phase of growth for both constructs, consistent with the postexponential nature of agr activation as described in previous studies (17, 31). More importantly, the level of fluorescence was significantly higher in the wild-type MW2 than in the $mgrA$ mutant for the 10-h culture and the overnight samples. This finding, in conjunction with those of RN6390 in a previous study (17), confirmed the positive regulation of agr by $mgrA$.

The role of $sarA$ in biofilm formation in $mgrA$ mutants. The global regulator SarA has been shown to be a positive regulator of biofilm formation in *S. aureus* (3, 41). As SarA is a positive regulator of agr (5) and $mgrA$ negatively modulates biofilm formation via agr , we wanted to determine if $sarA$, which does not regulate $mgrA$ and vice versa (16), would con-

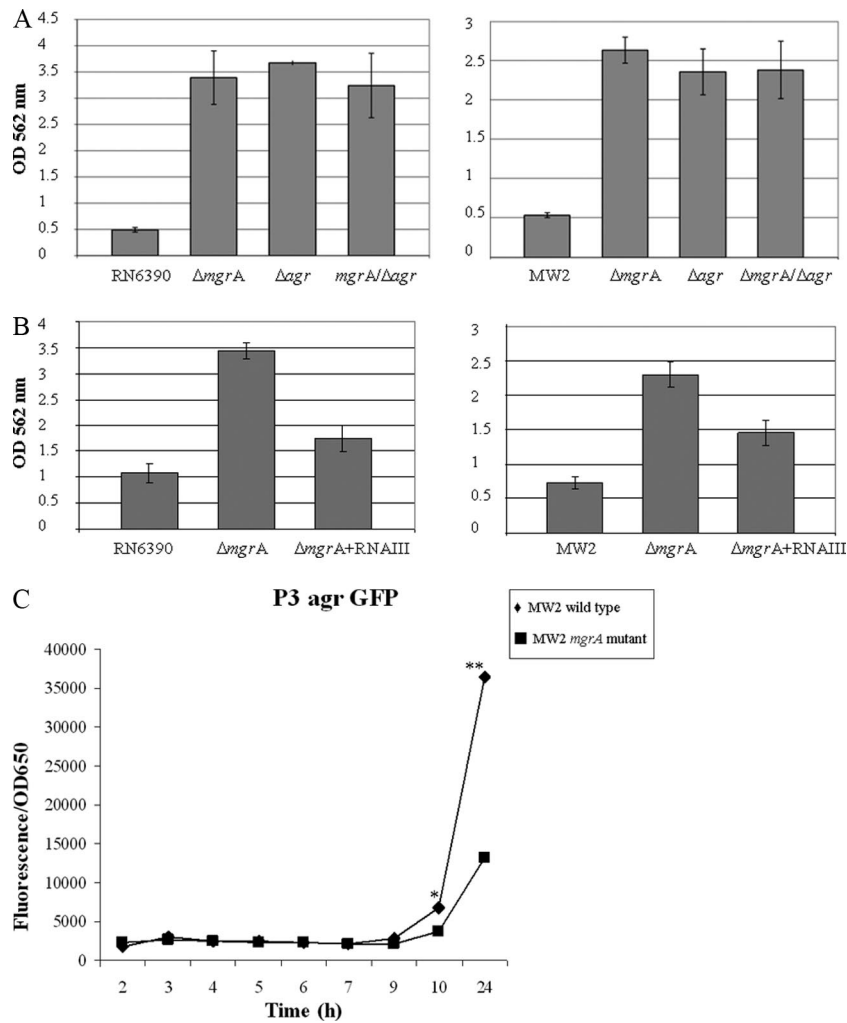


FIG. 6. Effect of RNAIII on biofilm formation in *mgrA* mutants of RN6390 and MW2. (A) *agr* deletion mutants were compared with *mgrA* mutants and double *mgrA agr* mutants for biofilm formation. Biofilms were quantitated by solubilizing the crystal violet in 30% glacial acetic acid and measuring the OD₅₆₂. Statistical values as determined by the Student *t* test were as follows: RN6390 versus the *mgrA* mutant, $P < 0.00001$; RN6390 versus the *mgrA agr* mutant, $P < 0.00015$; MW2 versus the *mgrA* mutant, $P < 0.0001$; MW2 versus the *agr* mutant, $P < 0.004$; and MW2 versus the *mgrA agr* mutant, $P < 0.00002$. There were no statistical differences among the *mgrA*, *agr*, and *mgrA agr* mutants in both RN6390 and MW2. (B) The plasmid pRN6735 with the *blaZ* promoter driving RNAIII was introduced into *mgrA* mutants of RN6390 and MW2 followed by quantitation of the biofilm formation as above, using parents and the *mgrA* mutant as controls. The *P* values were as follows: the *mgrA* mutant of RN6390 versus the *mgrA* mutant with RNAIII, $P < 0.0004$; RN6390 versus the *mgrA* mutant with RNAIII, $P < 0.02$; the *mgrA* mutant of MW2 versus the *mgrA* mutant with RNAIII, $P < 0.005$; MW2 versus the *mgrA* mutant with RNAIII, $P < 0.003$. (C) The plasmid pALC1743 with the RNAIII promoter driving the expression of GFP_{uvr} was introduced into MW2 and its isogenic *mgrA* mutant. Promoter activity was measured as fluorescence units per OD₅₆₂ over a 10-h period and then overnight. Each value represents the mean of three distinct clones from each genetic background. * and **, statistical significance by the Student *t* test when comparing fluorescence at 10 h ($P < 0.03$) and overnight ($P < 0.05$), respectively, between MW2 and the isogenic *mgrA* mutant.

tribute independently to biofilm formation in *mgrA* mutants. Accordingly, we assayed for biofilm formation first in *sarA* mutants of RN6390 and MW2 (Fig. 7A). With RN6390, which is a relatively poor biofilm former, a mutation in *sarA* did not lead to an appreciable difference in biofilm formation between the *mgrA* mutant and the parent. In the background of MW2, which is a slightly better biofilm producer, biofilm formation was reduced in *sarA* mutants versus the respective parents in concordance with previous studies (41); in contrast, the corresponding *mgrA* mutants exhibited elevated levels of biofilm formation. When the *sarA* mutation was introduced into the *mgrA* mutant of strain RN6390, the biofilm phenotype was

significantly reduced in the double mutant in comparison to the *mgrA* mutant, at an intermediate level between the *sarA* and *mgrA* mutant, while this effect was more pronounced in the MW2 background (Fig. 7A). This finding suggested that *sarA* is required for biofilm formation in *mgrA* mutants. Given the intermediate biofilm phenotype of the double mutant, the *sarA*-mediated effect on biofilm formation is probably independent of *mgrA* and also *agr*, since both the *mgrA* and *sarA* mutants have been shown to downregulate *agr*, but they displayed divergent biofilm phenotypes, with *sarA* being a positive regulator and *agr* being a negative regulator.

To further elucidate the contribution of *sarA* to the biofilms

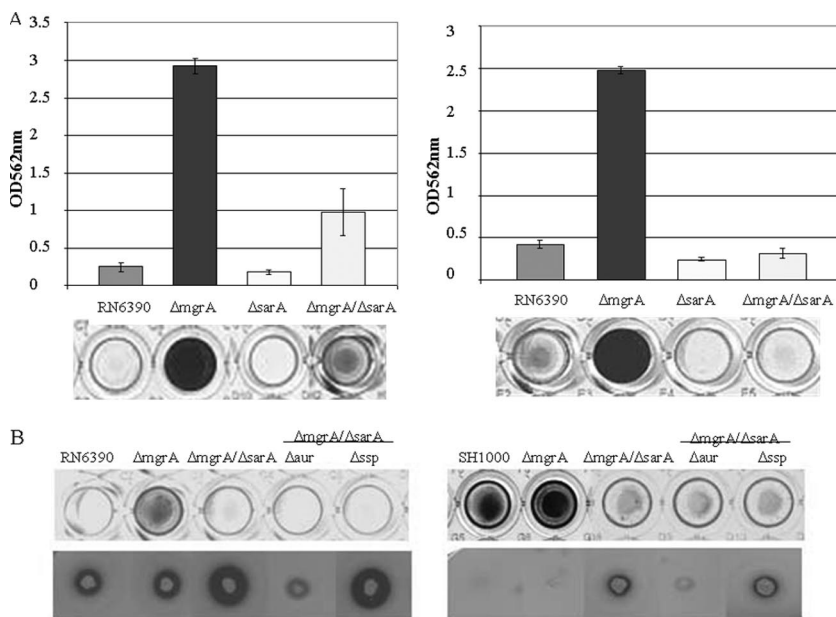


FIG. 7. (A) Analysis of biofilm formation in wild-type strains RN6390 and MW2, isogenic *mgrA* mutants, *sarA* mutants, and the double $\Delta mgrA \Delta sarA$ mutants. Significant differences were found between the *mgrA* mutants and the corresponding $\Delta mgrA \Delta sarA$ mutants. *P* values were as follows: RN6390 versus the *mgrA* mutant, $P < 0.002$; the *mgrA* mutant of RN6390 versus the double *mgrA sarA* mutant, $P < 0.02$; MW2 versus the *mgrA* mutant, $P < 0.004$; the *mgrA* mutant of MW2 versus the double *mgrA sarA* mutant, $P < 0.003$. The results of the microtiter plates are shown below. (B) Biofilm formation in microtiter plates of *mgrA* mutants, *sarA* mutants, $\Delta mgrA \Delta sarA$ mutants, and the $\Delta mgrA \Delta sarA \Delta aur$ and $\Delta mgrA \Delta sarA sspA$ triple mutants (top panels). The corresponding protease activity by these strains on skimmed-milk agar plates is shown in the bottom panels.

formed by *mgrA* mutants, we determined if decreased biofilm formation in *mgrA sarA* mutants may be due to the upregulation of protease activity typically found in *sarA* mutants. As displayed in Fig. 7B (bottom panels), protease production was increased in double *mgrA sarA* mutants compared with the isogenic parent RN6390. However, the introduction of an additional mutation in either the aureolysin (*aur*) or V8 protease gene (*sspA*) in the *mgrA sarA* mutant did not restore the biofilm phenotype to that of the single *mgrA* mutants (Fig. 7B, top panels), thus suggesting that the upregulation of these protease genes is not the major cause of diminished biofilm formation in *sarA mgrA* double mutants.

We also examined the role of *sigB* in biofilm formation in the *mgrA sarA* mutant of strain SH1000. In contrast to RN6390, SH1000 has a functional *rsbU* with intact *sigB* activity. Consistent with previous reports (6, 34), an intact *sigB* system in strain SH1000 increased biofilm production in comparison to RN6390 (Fig. 7B, right panels). Likewise, the *mgrA* mutant of SH1000 also exhibited enhanced biofilm formation compared to the parent. As with RN6390, a double *mgrA sarA* mutant of SH1000 almost completely eliminated biofilm production found in the single *mgrA* mutant. The inactivation of either *aur* or *sspA* in the double mutant did not restore biofilm formation to that of the single *mgrA* mutant in the SH1000 background. These results verified that the effect of *sarA* on biofilm formation in the *mgrA* mutant does not require full *sigB* activity.

DISCUSSION

MgrA is a 147-residue protein that belongs to a family of regulatory proteins called the SarA protein family by virtue of

their homology to SarA of *S. aureus* (5). Contrary to other SarA protein family members, MgrA is more similar to MarR of *E. coli* than to SarA of *S. aureus* (16). Phenotypically, MgrA is a negative regulator of autolysis (16) and a positive regulator of virulence determinants by controlling *agr* expression (17). Recent studies have linked biofilm formation to cell lysis mediated by *cidA* (35) and also to autolysis in *arlRS* mutants (12, 38). Recognizing that an *mgrA* mutant has an augmented autolytic phenotype (16), we explored and confirmed that a mutation in *mgrA* led to enhanced biofilm formation in strains RN6390, SH1000, and MW2, while the complemented mutants in all three genetic backgrounds restored the parental biofilm phenotype (Fig. 1). In contrast to previous studies on *S. aureus* biofilms (6, 21, 34), the biofilm formed by *mgrA* mutants is not dependent on *rsbU*, a positive regulator of *sigB* activity, as evidenced by the comparable biofilm formation between *mgrA* mutants of the *rsbU*-defective RN6390 and the *rsbU*-repaired SH1000 in our study. Surprisingly, our observation on enhanced biofilm formation in *mgrA* mutants contradicted the findings of Tu Quoc et al., who found, in a forward genetic screen, diminished biofilm production in an *mgrA* mutant, among others, in a pediatric *S. aureus* clinical isolate from the University of Geneva Hospital collection called strain S30 (40). The difference between our finding and that of Tu Quoc et al. is not immediately apparent, but it may be due to strain differences. However, it should be emphasized that our studies incorporate both well-characterized laboratory and clinical isolates, while *S. aureus* S30 is a previously uncharacterized clinical strain.

In a transcriptional profiling study of the *mgrA* regulon of *S.*

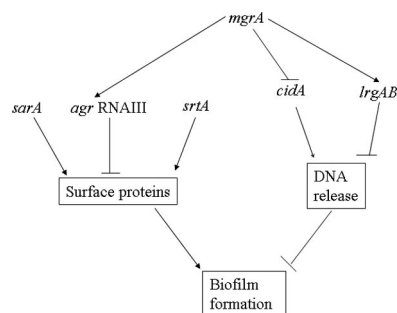


FIG. 8. The proposed model of biofilm formation in *mgrA* mutants. The inactivation of *mgrA* would reduce *agr* expression, enhancing surface protein expression, reducing nuclease secretion (23), and hence augmenting biofilm formation. A mutation in *mgrA* also increases CidA expression as well as reduces LrgA expression (23), thus promoting autolysis and extracellular DNA release to facilitate biofilm formation. SrtA may also affect surface protein expression. The mode of interaction between MgrA and SarA in biofilm formation remains to be defined.

aureus strain Newman (23), Luong et al. found that MgrA upregulates 175 genes while downregulating 180 others. Among these are the positive regulation of *lrgAB* and the negative regulation of *lytN* and *cidA* by MgrA. LytN is a murein hydrolase, while CidA and LrgA are analogous to those of bacteriophage holins and antiholins, respectively. The bacteriophage holin molecule facilitates cell lysis while the antiholin opposes this effect. Accordingly, the negative regulation of CidA and the positive regulation of LrgA by MgrA would reduce cell lysis and the concomitant release of extracellular DNA, which can serve as a component of *S. aureus* biofilm (18, 35, 37). In particular, programmed cell lysis mediated by CidA has been shown to release extracellular DNA to promote biofilm formation (35) (Fig. 8). In our study, the addition of DNase I to media containing *mgrA* mutants of RN6390 and MW2 prior to biofilm formation resulted in the complete eradication of biofilms after overnight growth. However, DNase I treatment (0.7 to 64 units/ml) of preformed biofilms had no significant attenuation effect. This latter finding differs from that of Izano et al. (18), who showed that established *S. aureus* biofilms can be dispersed by a high concentration of DNase I (100 μ g/ml or 40 units/ml). These differences cannot be explained by the DNase I dosage effect. However, it is conceivable that the biofilm formed by *mgrA* mutants may differ from the mature biofilms of wild-type *S. aureus* strains employed by Izano and colleagues (18). Nonetheless, extracellular DNA is probably an important part of the early biofilm in *mgrA* mutants of *S. aureus*. Once a biofilm of an *mgrA* mutant is formed, the efficacy of DNase I in disrupting the biofilm is less prominent, possibly due to a lack of access to DNA in the lower stratum of the biofilm. However, we did not entirely rule out the possibility that DNA may be a lesser component of the mature biofilm in *mgrA* mutants.

S. aureus can form biofilms that are both dependent on and independent of PIA, a polymer of amino sugar (8, 38, 41). However, a dot blot analysis with anti-PIA antibody as well as transcriptional fusion with an *ica* promoter driving *gfp_{uvr}* revealed that biofilms formed by the *mgrA* mutants are independent of the *ica* locus which encodes PIA. This was confirmed by

the finding that *ica* expression seemed to be unchanged or slightly decreased in *mgrA* mutants of RN6390 and MW2.

Besides DNA and PIA, we also evaluated the contribution of surface proteins to the initiation of biofilms in *mgrA* mutants. Accordingly, we deleted *srtA*, the sortase A gene, in *mgrA* mutants, which anchors almost all of the surface proteins to the peptidoglycan chain in *S. aureus* (29). Interestingly, the deletion of *srtA* in *mgrA* mutants of RN6390 and also MW2 reduced the formation of biofilms, indicating that the effect of the *mgrA* mutation on biofilms is mediated in part by upregulating surface protein expression. As the double *mgrA srtA* mutant has an intermediate biofilm phenotype compared with the *mgrA* mutant (Fig. 3) and *srtA* and *mgrA* are positive and negative contributors to biofilm formation, respectively, these data indicate that MgrA also represses biofilm formation independently of *srtA* in *S. aureus* (Fig. 8).

As an additional assay to evaluate the contribution of surface proteins in early biofilm and preformed biofilm formation in *mgrA* mutants, we also exposed both sets of biofilms to proteinase K treatment. Treatment of both early and preformed biofilms with proteinase K reduced biofilm formation in *mgrA* mutants of RN6390 and MW2 to near-parental levels, thus confirming the importance of surface proteins in the initiation and maintenance of biofilms. Based on these studies, we theorize that surface proteins may be important for early adhesion as well as cell-cell aggregation in late biofilm architecture.

SarA is a global transcription factor that enhances biofilm formation, activates the expression of many surface protein adhesins, and also represses the secretion of proteases in *S. aureus*. In concordance with other studies (3, 41), the *sarA* mutants of RN6390 and MW2 failed to form biofilms. Interestingly, the introduction of a *sarA* mutation into *mgrA* mutants led to significantly lower biofilm formation compared with the single *mgrA* mutants. Given that *sarA* does not regulate *mgrA* and vice versa (16), it is likely that *sarA* impacts genes involved in the biofilm phenotype (e.g., surface protein genes) downstream of *mgrA*. As the *sarA* mutants also express elevated levels of proteases, we also ascertained the role of proteases in reducing biofilm formation in *mgrA sarA* double mutants of RN6390 and MW2. As anticipated, the double *mgrA sarA* mutant exhibited enhanced proteolytic activity (Fig. 7B, bottom panels), which theoretically should cleave surface proteins necessary for attachment and/or cell-cell aggregation. However, inactivation of the aureolysin (*aur*) or the V8 protease gene (*sspA*) in the double *mgrA sarA* mutant did not restore biofilm formation to the double mutant. These data make it less likely for proteases to be the prominent mediator of reduced biofilm formation in the *mgrA sarA* double mutant. However, it should be stressed that *S. aureus* possesses other protease gene products, including acidic proteases and metalloproteases. Indeed, residual protease activity was still present in the triple *mgrA sarA aur* and *mgrA sarA sspA* mutants of RN6390 (Fig. 7B, bottom panels). Therefore, a definitive role for protease in biofilms cannot be assessed until the plethora of protease genes in *S. aureus* is completely inactivated.

The positive regulation of *agr* by the *mgrA* locus (17) (Fig. 6C), coupled with enhanced biofilm formation in *agr* mutants (32, 44) and *mgrA* mutants in this study (Fig. 6A), led us to examine the role of *agr* in biofilm formation in *mgrA* mutants.

As predicted, a double *mgrA agr* mutation in RN6390 and MW2 resulted in elevated biofilm production compared with the parents, similarly to what we have observed with the single mutants (Fig. 6A). The expression of RNAIII under the control of an exogenous *blaZ* promoter, which is not subjected to *mgrA* regulation, could reduce the biofilm formation of *mgrA* mutants but not to the level of the parents (Fig. 6B), thus suggesting that biofilms formed in *mgrA* mutants are attributable in part to reduced *agr* expression. This *agr*-mediated effect can be explained partially by the increased expression of surface protein adhesins attributable to reduced *agr* expression in early and established biofilms in *mgrA* mutants. In addition, recent transcriptional profiling studies have also demonstrated the reduced expression of nuclease (*nuc*) in the *mgrA* mutant (23). The reduction in secreted nuclease in *mgrA* mutants may conceivably expand the pool of extracellular DNA, a core component of early biofilms in these mutants. As *agr* is also a positive regulator of nuclease, it remains to be determined if the effect of *mgrA* on *nuc* is dependent on *agr*. Finally, the partial complementation by RNAIII in *trans* in *mgrA* mutants also implied that additional factors other than RNAIII likely play a role in biofilm formation in *mgrA* mutants.

In summary, MgrA has previously been shown to be a pleiotropic regulator of autolysis, virulence factors, and efflux activity in *S. aureus*. We have now added biofilm formation to that list. Based on our studies, it appeared that the biofilms formed by *mgrA* mutants are not dependent on PIA but instead rely on surface proteins (Fig. 8) and extracellular DNA for early formation and only on surface protein for the maintenance of mature biofilms. The biofilm formed by *mgrA* mutants is independent of SigB and selective proteases, such as V8 protease and aureolysin, but is reliant on *agr* and *sarA* (Fig. 8). Complementation data showed that the effect of *mgrA* on biofilms is likely mediated in part by regulating *agr*. However, the manner by which *mgrA* interfaces with *sarA* in biofilm formation is not due to *agr*, since SarA, as a positive regulator of *agr* (5), enhances biofilm formation while *agr* does not.

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