Correction

**MICROBIOLOGY**

The authors note that Fig. 2 appeared incorrectly. The corrected figure and its legend appear below.

![Corrected figure and its legend](www.pnas.org/cgi/doi/10.1073/pnas.1523895113)

**Fig. 2.** Stability of *mgrA* mRNA. (A) Stability in various chromosomal mutants. (B) RNAIII complementation of deletion mutations in *mgrA* UTR. mRNA stability expressed as half-life in minutes. The numbers in parentheses represent strain number. *P* < 0.05, **P** < 0.01 (unpaired two-tailed Student *t* test between Newman and each mutant, *n* = 3).

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RNAIII, the effector of the agr quorum-sensing system, plays a key role in virulence gene regulation in *Staphylococcus aureus*, but how RNAIII transcriptionally regulates its downstream genes is not completely understood. Here, we show that RNAIII stabilizes *mgrA* mRNA, thereby increasing the production of MgrA, a global transcriptional regulator that affects the expression of many genes. The *mgrA* gene is transcribed from two promoters, P1 and P2, to produce two mRNA transcripts with long 5′ UTR. Two adjacent regions of the *mgrA* mRNA UTR transcribed from the upstream P2 promoter, but not the P1 promoter, form a stable complex with two regions of RNAIII near the 5′ and 3′ ends. We further demonstrate that the interaction has several biological effects. We propose that MgrA can serve as an intermediary regulator through which agr exerts its regulatory function.

Staphylococcus aureus | RNAIII | MgrA | virulence regulation

Small regulatory RNAs (sRNAs) play an important role in gene regulation in both eukaryotes and prokaryotes. In bacteria, sRNAs typically act by base pairing with target mRNAs with limited or extended complementarity (antisense mechanism), but they can also modulate protein activity (1, 2). sRNAs commonly act on their target mRNA near the translation initiation site by base pairing to block—and in some cases promote—translation. Some sRNAs cause alterations in the secondary structure of the target mRNA, thereby enhancing or reducing mRNA degradation (3, 4).

*Staphylococcus aureus* is an important human pathogen that produces many virulence factors that are regulated by an equally impressive number of regulators (5, 6). A key regulatory system involved in virulence regulation in *S. aureus* is the *agr* quorum-sensing system. A 514-nt RNA, RNAIII, is the effector through which most genes in the *agr* regulon are regulated (7, 8). RNAIII is one of the largest known sRNAs that regulate gene expression via an antisense mechanism by base pairing with target mRNAs (5, 9). RNAIII is capable of forming a stable structure that is characterized by 14 stem-loop motifs and three long-distance interacting helices (9). Several examples of regulation by direct interaction between RNAIII and its target mRNAs have been reported (10–14). A common denominator in these regulatory interactions is the targeting of translation initiation sequences, through base pairing of the CU-rich loop domains of RNAIII with regions containing the Shine and Dalgarno (SD) sequence or AUG start codon of the target mRNA, resulting in negative control of translation (15, 16). A notable exception is the activation of Hla by RNAII, in which the interaction unmasks the SD sequence to facilitate translation of *hla* mRNA (10). Although a recent report shows that RNAII activates *map* expression, the mechanism of activation is unknown (17). More recently, RNAIII has also been shown to negatively control *sbi* translation through direct interaction with the translation initiation sequences of the *sbi* mRNA. However, in this case, three distant RNAIII domains that do not include the CU-rich loop domains have been shown to directly interact with three distinct sites in the *sbi* mRNA to repress *sbi* translation (18).

Rot is a pleiotropic regulator that affects many toxins and surface proteins (19). The finding that RNAIII represses *rot* mRNA translation has advanced the understanding of *agr* regulation (12, 13). However, RNAIII and Rot transcriptomes only partially overlap (19, 20). Thus, RNAIII repression of Rot cannot completely account for how RNAIII regulates the majority of its downstream genes. This led Geisinger et al. (12) and Boisset et al. (13) to postulate that RNAIII must interact with one or more pleiotropic regulators. Here, we report that RNAIII can also affect its downstream genes through another important *S. aureus* global transcriptional regulator, MgrA, which has been shown to affect more than 350 genes involved in virulence, antibiotic resistance, autolysis, and biofilm formation (21–25). Our results suggest that part of the MgrA regulon is affected by RNAIII.

**Results**

**mgra Gene Is Transcribed by Two Promoters.** We have previously identified the *mgra* gene (22). To map the *mgra* promoter, we performed primer extension experiments. We found three transcriptional start sites: at nucleotides −123, −301, and −303 upstream of the ATG start codon (Fig. S1), which closely agreed with the nucleotide −124 and −302 sites previously reported (21). Most likely, nucleotides −301 and −303 represent start sites from the same promoter with the former being the preferred start site. We then designated the promoters as P1 and P2, as shown in Fig. 1. To further define the promoters, we constructed promoter fusion to the *xylE* reporter gene in plasmid pLL38 with successive deletions of DNA extending from 368 bp upstream of the *mgra* ATG start codon. Our results showed that deletion of P2 reduced reporter activity by about 33–43%, whereas deletion of both promoters resulted in about 92% reduction (Fig. 1), indicating that both promoters were functional and that the predicted promoters are most likely correct. It should be noted here that the P2 promoter is embedded within the coding region of a divergently transcribed upstream gene encoding a putative ABC transporter.

**P2′ UTR Affects the Stability of *mgra* mRNA.** The 123-nt and 301/303-nt UTRs transcribed from the P1 and P2 promoters, respectively, are unusually long for bacterial mRNAs. For convenience, herein we refer to the region between the P2 and P1 transcriptional start sites as the P2 UTR and the region between...
Gene, we performed deletion analysis. As mRNA. To investigate the mechanism, domains of RNAIII contributed to and has an effect UTR in the mgrA ends mRNA by targeting P2 UTR. (CYL11346) mRNA by base paring at two regions (transcription (P2 UTR and that the interaction is specific. deletion alone (compare 11346 to mRNA and the 3 100bp Fig. S5 mRNA (Fig. 2 P2 UTR was syn- mRNA by interacting with transcript are lo- end 28 nt and the 3 coding sequence. Overlapping transcripts are derived from pTL3234 with a 450-bp insert containing both promoters and the 3 coding sequence, except pTL3271, which was derived from pTL3289 with an additional 124 bp of upstream DNA (dashed line; not to scale). Xyle activities were assayed in Newman. The promoter activities are expressed as percentages of the activity of the parent plasmid.

the P1 transcriptional start site and the AUG start codon as the P1 UTR. To determine whether sequences within the UTR affect the expression of the mgrA gene, we performed deletion analysis. As shown in Fig. 1, a deletion of 95 bp within the P1 UTR reduced the reporter activity by about 33%, whereas a deletion of 138 bp within the P2 UTR reduced by about 47%, suggesting that the sequences within the UTRs are important for mgrA transcription.

One mechanism whereby the 5' UTR could affect gene transcription is by influencing mRNA stability. To determine whether this mechanism is involved, we first constructed a 35-bp deletion in the P1 UTR (ΔP1 UTR) and a 138-bp deletion in the P2 UTR (ΔP2 UTR) in the chromosome. The mutants were tested for mgrA mRNA stability by real-time RT-PCR (qRT-PCR) upon rifampicin-mediated transcription arrest. We found that the ΔP2 UTR mutation significantly reduced the mgrA mRNA stability, whereas the ΔP1 UTR mutation had no significant effect on mRNA stability (Fig. 2).

RNAIII Stabilizes mgrA mRNA by Interacting with mgrA P2 UTR. The above results suggest that sequences in the P2 UTR are involved in stabilization of mgrA mRNA. To investigate the mechanism, we searched for known S. aureus sRNAs using IntRNA (26) and identified RNAIII as a potential sRNA that could interact with the UTR of mgrA mRNA by base paring at two regions (Fig. S2). Both the 33-nt and 14-nt regions in the mgrA transcript are located within the P2 UTR, whereas the two corresponding complementary regions in RNAIII are located at the ends of the molecule (the 5' end 28 nt and the 3' end 14 nt). To test whether RNAIII is involved in stabilizing mgrA mRNA by interacting with P2 UTR, we first compared the effect of agr deletion on mgrA mRNA stability in the wild-type, ΔP1 UTR, and ΔP2 UTR strains. The results showed that the deletion of agr (CYL11346) decreased the half-life of mgrA mRNA (Fig. 2A). Furthermore, the deletion of agr in the ΔP1 UTR strain reduced stability to about the same level as that of the agr deletion alone (compare 11346 to 12501) but had no effect on mRNA stability in the ΔP2 UTR strain (compare 1845 to 12502). These results suggest that agr has an effect on mgrA mRNA stability by targeting the P2 UTR. To further confirm that RNAIII of the agr system is involved in the regulation, we expressed RNAIII under the control of an anhydroretocycline (itc)-inducible promoter. As shown in Fig. 2B, the induction of RNAIII increased (though not statistically significant) the mgrA mRNA stability in the ΔP1 UTR mutant in which the P2 UTR is intact, whereas the stability in the ΔP2 UTR strain remained the same with or without RNAIII induction. Because the stability test above using qRT-PCR was unable to distinguish between P1- and P2-derived mgrA mRNA species, we further carried out Northern analyses and confirmed that agr affected only the P2-derived mRNA species (Fig. S3A). In addition, to test the effect in a different genetic background, we showed that agr deletion has similar effect in strain USA300 LAC (JE2), a strain with naturally high RNAIII level (Fig. S3B). These results further suggest that RNAIII is involved in stabilizing mgrA mRNA by targeting P2 UTR.

As shown in Fig. S1, the mgrA P2 promoter is located within the divergent NWGN_0656 coding sequence. Overlapping transcripts have been shown to be degraded by RNase III, thereby affecting mRNA level of the genes involved (27). Thus, it is possible that RNAIII could affect NWGN_0656 expression, thereby affecting mgrA mRNA levels. However, we showed that RNAIII had no effect on NWGN_0656 transcription (Fig. S4), indicating that RNAIII does not regulate mgrA mRNA levels through NWGN_0656.

mgrA UTR Interacts with RNAIII at Two Separate Domains. To determine whether the complementary regions identified above are involved in the interaction between RNAIII and mgrA P2 UTR, we used RNA-RNA EMSA. RNA corresponding to the mgrA P2 UTR was synthesized in vitro and labeled with biotin. Various regions of the RNAIII were also synthesized in vitro. Incubation of the labeled mgrA P2 UTR probe with full-length RNAIII resulted in a shifted band (Fig. 3A), which could be effectively competed away with 10- to 30-fold excess of cold fragment (lanes 2 and 3 from the right in Fig. 3A). An irrelevant RNA fragment derived from the 5'-end of cplC (negative control) did not result in a band shift. By using partial RNAIII fragments, we showed that deletions of the segments containing either one of the complementary regions in RNAIII still resulted in a shifted band, but deletions of both regions abolished the shift (Fig. 3A and Fig. S5). These results suggest that both the 28-nt and 14-nt regions of RNAIII contribute to the interaction of RNAIII with mgrA P2 UTR and that the interaction is specific.

To further confirm the interaction between RNAIII and mgrA mRNA using EMSA, we labeled RNAIII. As shown in Fig. 3B, fragments containing both the 33-nt and the 14-nt regions of mgrA UTR resulted in a shifted band. However, deletions of the 14-nt region, but not the 33-nt region, abolished the shift. These results did not totally conform to those of Fig. 3A and Fig. S5, in which both the 5' and the 3' domains of RNAIII contributed to the binding. However, close examination of the shifted bands revealed a much-weeker band intensity when the 14-nt region was deleted compared with that of the deletion of the 28-nt region (Fig. 3A and Fig. S5). Thus, based on these results, we speculate that the perfectly complementary 14-nt domain may play a more important role than the 28-nt/33-nt domain in the interaction between RNAIII and mgrA mRNA.

RNA SHAPE-Directed Chemical Probing of Interaction of mgrA 5' UTR with RNAIII. We then used the RNA SHAPE (selective 2'-hydroxyl acylation analyzed by primer extension) method (28) to analyze the mgrA UTR structure, with or without the presence of RNAIII. N-methylisatoic anhydride (NMIA) was used to form a 2'-O-adduct, with flexible nucleotides representing unpaired sequences, which were then detected by primer extension. The in vitro synthesized 303-nt mgrA UTR with adaptor sequences at both 5' and 3' ends was used as the template for the reaction and primer extension. The raw data were analyzed by QuShape software (28-30). This experimental design resulted in highly reproducible SHAPE reactivity data of the mgrA UTR (Fig. 4A). The reactivity data were then used to predict the structure of mgrA UTR (Fig. 4B) by using RNAstructure and XRNA software programs (31, 32). The proposed structure indicates some of the nucleotides in the 14-nt (nucleotides 7-20) of the P2 UTR are involved in the interaction between RNAIII and mgrA mRNA.

Fig. 2. Stability of mgrA mRNA. (A) Stability in various chromosomal mutants. (B) RNAIII complementation of deletion mutations in mgrA UTR. mRNA stability expressed as half-life in minutes. The numbers in parentheses represent strain number. *P < 0.05, **P < 0.01 (unpaired two-tailed Student t test between Newman and each mutant, n = 3).
SHAPE analysis of UTR in the absence (Gupta et al. UTR RNA. The sequences of 0.80 unit); black, unreactive (UTR. (Fig. 4
To locus when the S. aureus primer contains a portion of the 14-nt (nucleotides 447–460) and the 28-nt (nucleotides 8–35) domains of RNAIII, which were predicted to interact with mgrA, became less accessible to NMA. Few or no changes were found in the rest of the RNAIII sequences. We also modeled the RNAIII structure based on the reactivity data (Fig. S6b), which matches well with the structure of RNAIII predicted using chemical and enzymatic probing (9). The high similarity between our predicted RNAIII structure and the previously established structure indirectly validates our RNA SHAPE results. These results from SHAPE analyses are in line with our model that mgrA UTR interacts with RNAIII at the 14-nt and 33-nt/28-nt domains, which further corroborate the EMSA study described above.

RNA Walk Reveals in Vivo Interaction of RNAIII and mgrA UTR. To investigate the interaction of RNAIII and mgrA mRNA in vivo, we modified the RNA Walk method that had been used to study RNA–RNA interactions in trypanosomes (33). The method uses AMT (4-aminomethyl-trioxsalen hydrochloride) to induce cross-linking under UV irradiation, which links two RNA molecules in close proximity within the cell. The cross-linked molecules were then mapped by RT-PCR, in which the primers spanning the region of an interacting domain cannot be copied efficiently because of cross-linking. S. aureus Newman, CYL1844 (ΔP1 UTR), CYL1845 (ΔP2 UTR), and CYL11346 (ΔmgrA mutant) were treated with AMT and UV-irradiated. Total RNA from the strains, with or without treatment, were isolated, converted to cDNA, and then PCR-amplified using primers spanning the P2 UTR containing the 14-nt region (the 5′ primer contains a portion of the 14-nt region sequence for specific amplification of the interacting region) (Table S1). Amplification of the lu gene was included as a control. As shown in Fig. 5A, a 170-bp PCR fragment could be detected in strains with an intact P2 UTR and an intact agr locus when the cultures were not treated. However, no PCR product was detected in treated cultures (Fig. 5A, lanes 1–4). On the other hand, the 170-bp PCR fragment was detected in the agr-deleted strain but not in the ΔP2 UTR strain, with or without AMT and UV (Fig. 5A, lanes 5–8). In addition, treatment did not affect amplification of the lu gene in any strain. These results demonstrated that the presence of both agr

96–109) and the 33-nt (nucleotides 57–89) regions are involved in the formation of secondary structures. However, the majority of the nucleotides in these regions are moderately reactive (Fig. 4A, suggesting that the hybrids are not stable. These regions also became less accessible to NMA upon RNAIII addition (Fig. 4A, Lower, and Fig. 4C), suggesting that RNAIII displaces the hybrids to form more stable duplexes at these regions. Besides the changes in reactivity at the RNAIII-interacting regions, we found little reactivity change in the rest of the mgrA UTR molecule except two small regions: one near the 5′-end (nucleotides 23–27) and the other near the 3′-end (nucleotides 263–278). The former is near the regions that interact with RNAIII suggesting that the interaction with RNAIII induces some changes in secondary structure at nearby sequences. The latter includes the region that is predicted to form a long-distance hybrid with the 33-nt domain within the mgrA UTR (Fig. 4B) and, therefore, the increase of reactivity at this region is consistent with the notion that these sequences are displaced upon RNAIII interaction.

Similarly, we analyzed the RNAIII structure by RNA SHAPE in the presence or absence of mgrA UTR (Fig. S6A). In the presence of mgrA UTR, we found the 14-nt (nucleotides 447–460) and the 28-nt (nucleotides 8–35) domains of RNAIII, which were predicted to interact with mgrA, became less accessible to NMA. Few or no changes were found in the rest of the RNAIII sequences. We also modeled the RNAIII structure based on the reactivity data (Fig. S6b), which matches well with the structure of RNAIII predicted using chemical and enzymatic probing (9). The high similarity between our predicted RNAIII structure and the previously established structure indirectly validates our RNA SHAPE results. These results from SHAPE analyses are in line with our model that mgrA UTR interacts with RNAIII at the 14-nt and 33-nt/28-nt domains, which further corroborate the EMSA study described above.

Fig. 3. RNA EMSA. (A) Labeled mgrA P2 UTR reacted with various deletions of 514-nt RNAIII. cplC RNA was used as a negative control. (B) Labeled RNAIII reacted with various deletions of mgrA UTR RNA fragments A through F. Red and green boxes represent matched sequences in each RNA species. Triangles indicate transcrptional start sites.

Fig. 4. SHAPE analysis of mgrA UTR. (A) Average reactivity of each nucleotide of mgrA UTR in the absence (Upper) or presence (Lower) of RNAIII. Red, highly reactive (>0.80 unit); orange, moderately reactive (0.40–0.80 unit); black, unreactive (<0.40 unit), n = 2. (B) Predicted structure of mgrA UTR RNA. The sequences of the 14-nt domain are in red and the 33-nt domain in green. Solid and open arrowheads indicate increase and decrease, respectively, in nucleotide reactivity in the presence of RNAIII. (C) Comparison of reactivity of each nucleotide in the RNAIII-binding domains in mgrA P2 UTR. Red trace, with RNAIII; Blue trace, without RNAIII.
RNAIII and the P2 UTR was required to form a cross-link adduct, suggesting their specific interaction in vivo.

**Mutation in the 14-nt Site Confirms Interaction of RNAIII and mgrA UTR.** To further demonstrate that the RNAIII interacts with mgrA mRNA, we constructed a 5-bp substitution mutation within the 14-bp domain, in the Newman chromosome. These 5 bp were chosen because of their high GC content and relative central location in the 14-nt domain (Fig. S2C). The mutation did not affect bacterial growth (Fig. S7). We then assessed whether the mutation affected mgrA mRNA stability. As shown in Fig. 2, the 5-bp mutation (CYL12916) resulted in a significant reduction in mRNA stability, similar to that of the agr deletion. These results suggest that the 14-nt domain in the P2 UTR is critical for mgrA mRNA stability. Because the 5-bp mutation within the 14-bp domain cannot be complemented with a plasmid, we sought to revert the mutation to the wild-type sequence to ensure that the mutant phenotype was not a result of inadvertent secondary mutations during the construction of the mutant. The mgrA mRNA stability of the revertant (CYLA22) was similar to that of the Newman (Fig. 2A), indicating that the mutant phenotype was a result of the 5-bp mutation.

As the 14-nt domain affects stability of mRNA transcribed from the P2 promoter, but not from the P1 promoter, we expected that MgrA production would be partially affected by the mutation in the 14-nt domain or the agr regulatory system under our experimental conditions. By Western analyses, we found that the amounts of MgrA were similar between the 5-bp mutation mutant and the Δagr mutant, which were both about 60% of the wild-type (Fig. 5B), suggesting that agr affects mgrA expression through the 14-bp domain in the P2 UTR. As expected, the revertant produced a similar amount of MgrA as Newman and the ΔmgrA mutant produced, essentially, an undetectable amount of protein.

To directly confirm the interaction, we constructed a 5-bp mutation in RNAIII such that the 14-nt region of the RNAIII is complementary to the 5-bp mutation in the 14-nt region of mgrA mRNA in strain CYL12916 described above. The 5-bp mutant RNAIII fragment was cloned in pML100 under an aTc-inducible promoter (pRGE23). As shown in Fig. 5B, the mutation in RNAIII restored the MgrA protein production in CYLA15 (CYL12916 Δagr) to a similar level produced in Newman. These results strongly suggest that RNAIII directly interacts with mgrA mRNA at the 14-nt domain to stabilize the mgrA mRNA stability.

**Phenotypic Effects of a 14-nt Domain Mutation in mgrA P2 UTR.** MgrA has been shown to affect capsule, surface proteins, toxins, autolysis, and antibiotic resistance (21–24, 34). To determine whether RNAIII-mgrA mRNA interaction has an effect on biological function, we tested a phenotype of CYL12916 containing the 5-nt mutation in the mgrA UTR. We found that hla, which encodes α-toxin, was affected by the 5-nt mutation to the same degree as the agr deletion (Fig. 6A), suggesting that agr activates hla primarily through mgrA in Newman. We also found that abcA, which encodes an ABC transporter involved in antibiotic resistance (34), and capD, which is one of the capsule genes in the cap operon, were significantly affected by the 5-nt mutation but not as drastically as the agr deletion (Fig. 6A), suggesting that agr affects these genes, in part, through interaction with the mgrA mRNA. Expression of all these genes was restored to the wild-type level in the 5-nt revertant strain. In addition, we also tested the effect of the 5-nt mutation on hemolysin activity and capsule production. As shown in Fig. 6A, mutation in agr reduced the hemolysin activity to an undetectable level, whereas the 5-nt mutation in the mgrA P2 UTR had a moderate reduction, which is consistent with a previous study that agr could affect hla at the translational level (10). On the other hand, the effect of the mutation on capsule production was comparable to the transcriptional effect measured by qRT-PCR.

Mgra has been shown to inhibit cell lysis (24). As shown in Fig. 6B, we found increased Triton X-100-induced cell autolysis in CYL12916 compared with Newman. Deletion of agr (CYLI1346) increased the autolysis to the same extent as the 5-bp mutation, suggesting that agr could affect autolysis through the 14-nt element. The 5-bp mutant of the 14-bp domain had a similar level of autolysis in Newman. To test this further, we complemented an agr deletion mutant (CYLI13056) with pLL48 expressing either intact RNAIII (pLL48-RNAIII) or mutated RNAIII (pLL48-RNAIIIm9, which contains a substitution of 9-nt in the 14-nt domain in RNAIII). Our results (Fig. 6C) showed that pLL48-RNAIIIm9 could not complement the agr deletion, whereas pLL48-RNAIII complemented the deletion although not to the level of Newman. These results suggest that RNAIII affects autolysis by stabilizing mgrA mRNA through the 14-nt domain.

These results demonstrated that the stabilization of mgrA mRNA by RNAIII could lead to significant biological effects. However, we found that the minimum inhibitory concentration (MIC) of oxacillin or ciprofloxacin was not affected by the 5-nt mutation, although others have reported that mgrA mutants have reduced MIC to ciprofloxacin (23) and that overexpression of mgrA increased the MIC to oxacillin in strain N315 (35). Thus, not all phenotypic changes associated with MgrA regulation are affected by RNAIII.

**Discussion**

Microarray studies showed that Agr and MgrA have similar effects on many virulence genes, suggesting that the two regulators may coregulate a subset of target genes (25). Here we found that RNAIII directly interacts with the mgrA mRNA. Our results suggest that RNAIII stabilizes the mgrA mRNA primarily through base pairing between the two RNA molecules at two domains. We provide several lines of evidence supporting this conclusion. RNAIII, as a regulatory effector of the agr system, has so far been shown to affect its target genes at the translational level by RNA base pairing that either masks or unmasks the translation initiation site (10, 11, 13, 14).

To our knowledge, the findings presented here are the first to show that S. aureus RNAIII affects a target gene by mRNA stabilization. The agr system regulates its targets mostly through an RNAIII-dependent pathway, but direct transcriptional activation of the psm genes by AgrA, the response regulator of the agr system, has been reported (8). Among the target genes, rot is the only regulatory gene found to be directly targeted by RNAIII. As such, Rot serves as an intermediate regulator of RNAIII (12, 13). In this study, our findings strongly suggest that MgrA is another intermediary regulator of RNAIII. However, we found that RNAIII only affected the transcript initiated from the P2 promoter, indicating that it has only partial control on mgrA expression. Interestingly, in addition to the P2 UTR, expression of mgrA is also affected by the sequences within the P1 UTR, suggesting a different regulatory factor (or factors) is involved (Fig. 1). A recent report has shown that RsA, a SigB-dependent sRNA, represses translation of MgrA by
blocking the SD sequence and the initiation codon of mgrA mRNA (36). This regulatory mechanism is unlikely to explain what we observed in the P1 UTR deletion because the deletion is further upstream of the SD sequence and its effect is opposite to that of RsA. Likely, there exists another regulatory element that could interact with P1 UTR. Thus, taken together, we envision that MgrA production could be influenced by multiple signals in which transcripts initiated from the P2 promoter could be controlled by quorum sensing through RNAIII, whereas transcripts from both promoters could be controlled by different stimuli by interacting with RsA through SigB, as well as an unknown regulatory element. The various modes of regulation at the mRNA level and the fact that the regulatory function of MgrA can be modulated by oxidation or phosphorylation at a conserved cysteine residue (37–39) indicate that MgrA regulatory function is likely modulated by multiple signals. Because MgrA has been shown to affect more than 350 genes and plays an important role in virulence regulation (25, 37, 40, 41), it is not surprising that the activity of MgrA is affected by multiple elements. This would allow MgrA to achieve optimal regulation of its regulon in response to various environmental conditions.

Although RNAIII regulation of MgrA had no effect on resistance to oxacillin or ciprofloxacin, we found that the regulation could lead to other biological effects. The effect of the 5-nt mutation in the mgrA 14-nt domain on capD and abcA expression and capsule production is less than that of the agr mutation, suggesting that agr could regulate these genes through alternative pathways. On the other hand, the effect of the 5-nt mgrA mutation on hla expression and Triton X-100-induced autolysis was comparable to that of the agr deletion alone, suggesting that agr may regulate hla and autolysis primarily through stabilization of mgrA mRNA. However, it has been shown that RNAIII can activate hla transcription through Rop regulation (12, 13), which was not revealed in our results. This discrepancy could be because of differences in experimental conditions or strains used. Additionally, RNAIII could bind to hla mRNA and activates hla translation (10), which agrees with our finding that agr deletion had more effect on α-toxin production than the 5-nt mgrA mutation. Thus, it is apparent that not all genes within the MgrA regulon are regulated by agr through mgrA. The 5-nt mutation resulted in about a 40% reduction in MgrA protein production. Likely, those genes in the MgrA regulon that are responsive to RNAIII may require a greater quantity of MgrA for regulation and that regulation of genes that are not responsive requires less MgrA. These results suggest that the regulation by RNAIII through MgrA could fine-tune MgrA regulation in response to quorum-sensing signals. The biological significance of this fine regulation, however, requires further investigations. Our studies further underscore the complexity of virulence gene regulation in S. aureus.

Our SHAPE analyses demonstrate that mgrA UTR directly interacts with RNAIII at the two short regions. Importantly, our RNAIII structure prediction is in close agreement with the published RNAIII structure by Benito et al. (9), with only minor differences, which validates our SHAPE methodology. Based on the predicted structure, the 14-nt sequence and the adjacent 33-nt sequence of the mgrA UTR are not totally unpaired in the absence of RNAIII. However, many of the nucleotides within these regions are moderately reactive, and some are highly reactive, suggesting they can readily form hybrids with RNAIII to form stable complexes when RNAIII is present. By using the RNA-Fold RNAhybrid (bibiserv.techfak.uni-bielefeld.de/rnahybrid) program, we calculated that the predicted hybridization minimum free energy of the complex between the 14-nt domains in the mgrA P2 UTR and RNAIII is −18.8 kcal/mol, whereas that of the 33-nt/28-nt is −25 kcal/mol. In comparison, the predicted minimum free energy of RNAIII complexes with its known RNA targets ranges from −15 to −34.5 kcal/mol (13). Thus, the estimation of free energy suggests that these complexes are quite thermodynamically stable.

RNAIII is a large sRNA with 14 stem-loops in which the C-rich sequences in H7, H13, and H14 hairpin loops have been shown to be the primary regulatory sites that interact with target mRNAs of several genes (15). However, a large region at the 5’ end of RNAIII including the H2 hairpin loop has been shown to interact with hla mRNA (10). More recently, it was demonstrated that three distant RNAIII domains—none of which involve the loop structures H7, H13, or H14—were involved in the regulation of sbi by agr (18). In this study, we showed that the sequences of the 14-nt at the 3’ end and the 28-nt at the 5’ end of RNAIII were involved in the interaction with the mgrA mRNA P2 UTR. These sequences are not within the loop domains of RNAIII and have not been previously shown to be involved in regulation, although they are directly adjacent to two of the three domains involved in sbi regulation (18). The regions located near both ends of the RNAIII molecule have been proposed to be involved in a long-distance interaction forming a helix (Helix C). However, our RNA SHAPE analysis (Fig. S6) indicated that Helix C is unstable, in agreement with the finding of Benito et al. (9). Nonetheless, because the 14-nt and 33-nt domains are juxtaposed in the mgrA UTR, it is tempting to speculate that the unstable Helix C of RNAIII may serve to bring the two distant domains of RNAIII close together to promote efficient interaction with mgrA UTR. Based on the results presented herein, we propose a model depicted in Fig. S8. We hypothesize that the 3’ RNAIII domain of
14-nt first interacts with the 14-nt sequence of {\it mgrA} UTR by base pairing. After the initial interaction, the 28-nt 5\' RNAII domain then pairs with the 14-nt UTR 33-nt sequence. In Newman, {\it mgrA} mRNA is expressed at the highest level in postexponential phase, at which time RNAII is most abundant (7, 42), allowing for pairing to occur readily. The complex then stabilizes {\it mgrA} mRNA, likely by blocking degradation by one or more RNases, leading to more MgrA protein production, thereby affecting expression of a subset of genes in the MgrA regulon.

Materials and Methods

Strain and Plasmid Construction. Strains and plasmids used in this study are listed in Table S2. Plasmid and strain construction were carried out using standard molecular methods. For details, see SI Materials and Methods. Primers used in this study are listed in Table S1.

mRNA Stability Assay. RNAs from transcription-arranged cultures were isolated at different time points. mRNA stability was assayed by qRT-PCR or Northern analysis. Half-lives were calculated by using linear regression analysis. For detailed procedures, see SI Materials and Methods.

RNA SHAPE Reactions and Analysis. RNA SHAPE experiments were performed according to the previously described method using NMIA (Sigma) (43, 44). The detailed procedures are provided in the SI Materials and Methods.

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RNA EMSA. RNA EMSA reactions were performed using RNAs synthesized by in vitro transcription. For details, see SI Materials and Methods.

RNA Walk. RNA Walk was performed essentially as described previously (33). Cross-linking of paired RNAs in vivo was done by adding AMT to cultures and UV exposure. RT-PCR was performed to detect cross-linked RNA regions. The detailed procedures are provided in the SI Materials and Methods.