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The *sagA/pel* locus does not regulate the expression of the M protein of the M1T1 lineage of group A *Streptococcus*

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Altered expression of Group A *Streptococcus* (GAS) virulence factors, including the M protein, can result as a consequence of spontaneous genetic changes that occur during laboratory and animal passage. Occurrence of such secondary mutations during targeted gene deletion could confound the interpretation of effects attributable to the function of the gene being investigated. Contradicting reports on whether the *sagA/pel* locus regulates the M protein-encoding *emm* might be due to inconsistent occurrence of mutations unrelated with *sagA*. This study examined the possibility that altered *emm* expression observed in association with *sagA/pel* deletion mutants is artifactual. *sagA* deletion mutants (MGAS2221Δ*sagA*) of M1T1 isolate MGAS2221 obtained using liquid broth for GAS growth during the deletion process had diminished *emm* transcription and no detectable M protein production. In contrast, a Δ*sagA* mutant of another closely genetically related M1T1 isolate had normal *emm* expression. The s*agB* gene does not regulate *emm*; however, one of three MGAS2221Δ*sagB* mutants had diminished *emm* expression. The *emm* regulator *mga* was downregulated in these M protein expression-negative strains. These results argue that *sagA* deletion does not directly cause the downregulation of *emm* expression. Indeed, two MGAS2221Δ*sagA* mutants obtained using agar plates for GAS growth during the deletion process both had normal *emm* expression. We conclude that the *sagA/pel* locus does not regulate *emm* expression in the M1T1 lineage and provide a protocol for targeted gene deletion that we find less prone to the generation of mutants exhibiting downregulation in *emm* expression.

Introduction

Group A *Streptococcus* (GAS) is a major human pathogen that causes various infections, ranging from uncomplicated infections of the pharynx and skin to life-threatening necrotizing fasciitis and streptococcal toxic shock syndrome. GAS produces many virulence factors to mediate its pathogenesis.[1] The M protein is a major surface protein, virulence factor, and protective antigen and is required for the resistance of GAS to phagocytosis by neutrophils.[2] Thus, downregulation of *emm* expression commensurately reduces the capacity of GAS to resist killing by neutrophils and to survive in non-immune blood. The *emm* gene is positively regulated by the virulence regulator Mga.[3] Another important virulence factor is streptolysin S (SLS) that contributes to the resistance of GAS to phagocytic clearance and skin and soft-tissue infections.[4-7] SLS is a bacteriocin-like toxin consisting of a posttranslationally modified peptide and activity-essential carrier molecules.[8] SLS is responsible for the β-hemolytic activity of GAS colonies grown on blood agar media and also has potent cytolytic activities to leukocytes, platelets, and subcellular organelles. The gene locus for SLS biosynthesis consists of nine genes (*sagA* to *sagI*).[9] SagA encodes pro-SLS peptide, which is posttranslationally modified to active SLS by SagBCD.[8]

There are contradicting reports as to whether the *sagA* locus has a regulatory role in the expression of the *emm* gene, which encodes the M protein. Betschel et al.[10] reported no detrimental effect on M protein production of a transposon insertion at the *sagA* locus in serotype M1 and M18 GAS. However, Li et al.[11] found that a serotype M49 GAS mutant with the insertion of a transposon in the promoter region of the *sagA* gene had downregulated expression of *emm* and several other virulence factors and designated the locus as *pel* for the pleiotropic effect locus. Mangold et al.[12] subsequently reported evidence that the *sagA/pel* locus in the M1 strain SF370 encodes a regulatory RNA. In contrast, Datta et al.[13] and Perez et al.[14] reported that deletion of the *sagA/pel* locus in M1 isolates does not cause alteration of virulence gene expression.

GAS can undergo variations in laboratory passages, resulting in variants that usually show reduced expression of M protein and other virulence factors.[15,16] It is also known that bacteria

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frequently accumulate mutations in response to nutritional stress during the stationary phase.[17] Various investigations of natural populations of GAS have revealed a high frequency of spontaneous mutations occurring in multiple global regulatory genes, including *mga*, *covRS*, and *ropB* that can mediate pleiotropic effects in GAS secreted and surface virulence factors.[18-21] The occurrence of a spurious mutation in one of these global regulators could alter the expression of multiple GAS virulence factors. We present evidence consistent with the hypothesis that the inconsistent finding of downregulation of *emm* expression in association with targeted *sagA*/*pel* deletion may be due to spontaneous mutations or phase variations that arise during laboratory manipulations. We conclude that the *sagA*/*pel* locus does not have a role in regulation of *emm* expression in the M1T1 lineage and have doubts about its capacity to regulate *emm* expression in GAS of other serotype lineages.

Results

Inconsistent results on effect of *sagA*/*pel* deletion in M1T1 GAS isolates on *emm* expression

For a separate study we generated a number of gene deletion mutants of two M1T1 isolates MGAS5005 and MGAS2221, including *sagA* gene deletion mutants. Our antibiotic selection marker-free MGAS2221Δ*sagA* and other mutants were generated in two steps using a suicide plasmid that contained the chloramphenicol resistance gene and a DNA insert of joint flanking fragments of target genes, as previously described.[22] In the first step, the suicide plasmid was integrated into the MGAS2221 chromosome through a single crossover between one of the *sagA* flanking fragments on the plasmid and its homologous sequence on the chromosome, resulting in a derivative strain that was resistant to chloramphenicol. In the second step, the chloramphenicol-resistant strain was grown in Todd-Hewitt broth supplemented with 2% yeast extract (THY) for at least 8 serial passages to allow for a second crossover between the other *sagA* flanking fragment on the plasmid and its homologous sequence on the chromosome, yielding *sagA* deletion mutants (MGAS2221Δ*sagA*). One passage equaled to the growth from an optical density at 600 nm (OD_{600}) of 0.05 to the late log growth phase with an OD_{600} of about 0.7 during day and the growth from OD_{600} of 0.05 to the late stationary growth phase with settled bacteria to the bottom of culture tubes during night (OD_{600} at the stationary phase in THY was about 0.9). The deletion mutants were screened by PCR analysis and confirmed by the lack of β-hemolytic activity on blood agar plates (**Fig. 1A and B**). Because we have previously found that some deletion mutants we generate in GAS exhibit reduced M protein production, we now routinely assess the production of the M protein in any GAS mutant that we generate by western blot analysis. The M protein was detected in western blots for MGAS2221 but not for nine MGAS2221Δ*sagA* mutants from two independent experiments. The western blotting results for two MGAS2221Δ*sagA* mutants (MGAS2221Δ*sagA*-1 and MGAS2221Δ*sagA*-2) are shown in **Figure 2A**. Real-time reverse transcription PCR analysis found that the levels of the M protein gene (*emm*) transcript in MGAS2221Δ*sagA*-1 and MGAS2221Δ*sagA*-2 were >40-fold lower than that in MGAS2221 (**Fig. 2B**), indicating that the downregulation of the M protein production was at the transcriptional level. These results were surprising to us since the *sagA* deletion in MGAS2221 did not affect gene expression of MGAS2221 in the study of Perez et al.[14] Puzzlingly, a MGAS5005Δ*sagA* mutant obtained from MGAS5005 using the same procedure had similar M protein production and *emm* transcription as the parent strain MGAS5005 (**Fig. 2**). Thus, as in the literature, we obtained conflicting results on the effect of *sagA* deletion on *emm* expression.

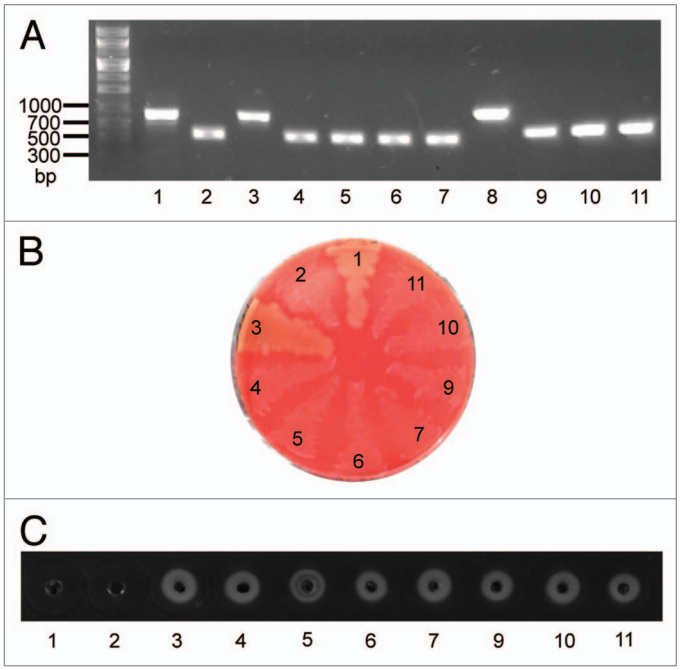

Figure 1. (**A**) PCR confirmation of *sagA* and *sagB* deletions in MGAS2221 and MGAS5005. Lanes 1 to 7 are PCR products for *sagA* deletion, and lanes 8 to 11 are PCR products for *sagB* deletion. (**B**) Loss of β-hemolytic activity in *sagA* and *sagB* deletion mutants. (**C**) *sagA* and *sagB* deletions had no effect on the SpeB protease production measured as the SpeB activity in the supernatant of overnight cultures. Numbers 1 through 11 in all the panels represent the following strains used in the analyses: 1, MGAS5005; 2, MGAS5005Δ*sagA*; 3, MGAS2221; 4, MGAS2221Δ*sagA*-1; 5, MGAS2221Δ*sagA*-2; 6, MGAS2221Δ*sagA*-3; 7, MGAS2221Δ*sagA*-4; 8, MGAS2221; 9, MGAS2221Δ*sagB*-1; 10, MGAS2221Δ*sagB*-2; and 11, MGAS2221Δ*sagB*-3.

MGAS5005 and MGAS2221 are representative isolates of a prevalent M1T1 clone from an invasive case in Ontario and a scarlet fever patient in Australia, respectively.[23] The two isolates have nearly identical genomes, differing by 7 synonymous and 9 non-synonymous single nucleotide polymorphism and an IS element in MGAS5005. In addition, MGAS5005 has a 1-bp deletion at base 83 of the *covS* gene of the two-component regulatory system CovRS (also known as CsrRS)[24] and consequently displays a higher virulence in mouse infection than MGAS2221,[25,26] which has the wild-type *covS* gene. We hypothesized that the difference in effect of *sagA* deletion on *emm* expression observed between MGAS2221 and MGAS5005 might be related to their difference in *covS* alleles. To investigate this possibility, we first transformed MGAS5005Δ*sagA* with a plasmid that contained the

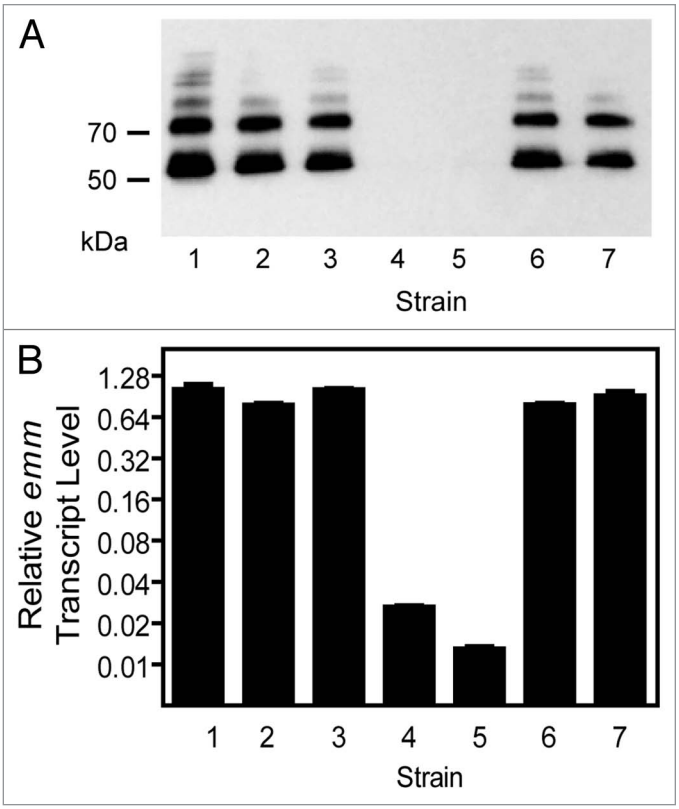

Figure 2. Inconsistent effect of *sagA* deletion on *emm* expression in MGAS5005 and MGAS2221. Western blot detecting the production of the M protein (**A**) and relative levels of *emm* transcript (**B**) in the following strains: 1, MGAS5005; 2, MGAS5005Δ*sagA*; 3, MGAS2221; 4, MGAS2221Δ*sagA*-1; and 5, MGAS2221Δ*sagA*-2; 6, MGAS2221Δ*sagA*-3; 7, MGAS2221Δ*sagA*-4. Strains 2, 4, and 5 were obtained using THY broth for GAS growth, and strains 6 and 7 were obtained using THY agar plates for GAS growth during the deletion process. Levels of *emm* mRNA were normalized to that of corresponding wild-type strain.

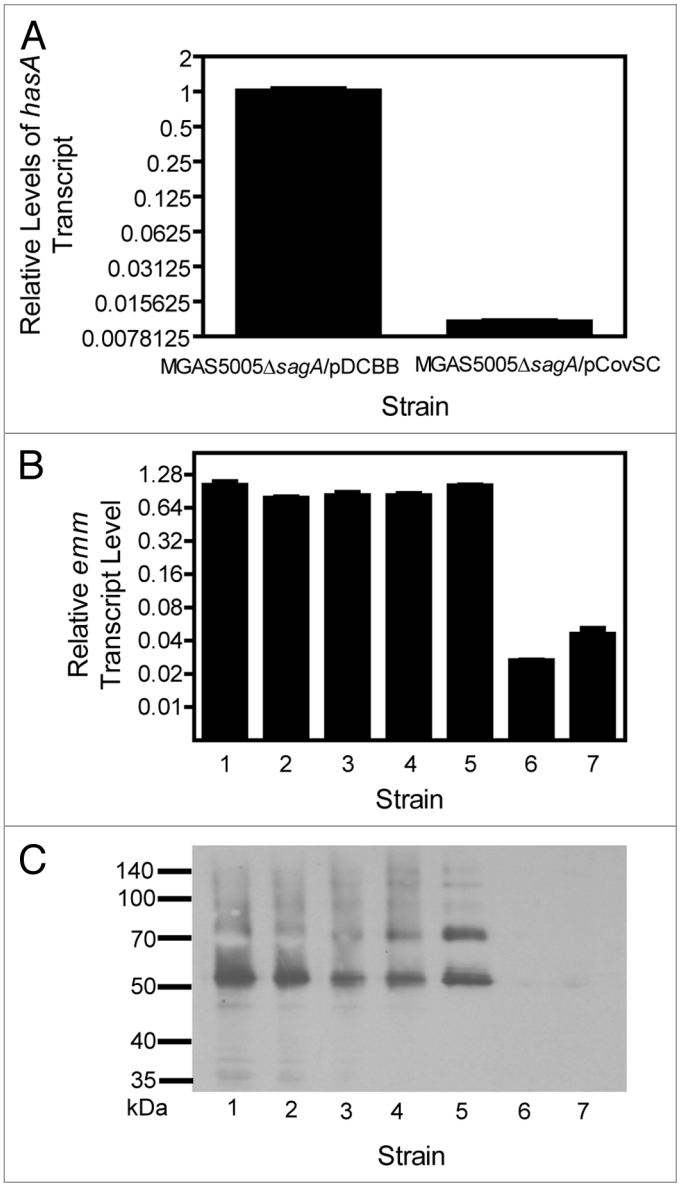

Figure 3. Inconsistent effect of *sagA* deletion on *emm* expression was not related to the functionality of the *covS* gene in MGAS5005 and MGAS2221. (**A**) Relative levels of hasA mRNA in MGAS5005Δ*sagA*/pDCBB and MGAS5005Δ*sagA*/pCovSC. (**B and C**) Relative levels of *emm* transcript (**A**) and western blot detecting the production of the M protein (**B**) in the following strains: 1, MGAS5005; 2, MGAS5005Δ*sagA*; 3, MGAS5005Δ*sagA*/pCovSC; 4, MGAS5005Δ*sagA*/pDCBB; 5, MGAS2221; 6, MGAS2221Δ*sagA*-1; and 7, MGAS2221Δ*sagA*Δ*covS*. Levels of *emm* or *hasA* mRNA were normalized to that of corresponding control strain.

wild-type *covS* gene (MGAS5005Δ*sagA*-pCovSC) and deleted the *covS* gene of MGAS2221Δ*sagA*-1 (MGAS2221Δ*sagA*Δ*covS*). Next, we measured *emm* transcription and M protein production in MGAS5005Δ*sagA*-pCovSC and MGAS2221Δ*sagA*Δ*covS*. The introduction of the wild-type *covS* gene in MGAS5005Δ*sagA* decreased the transcription of the *hasA* gene by 99% (**Fig. 3A**), indicating that in-trans expression of the wild-type *covS* gene restored the function of CovRS in MGAS5005Δ*sagA*. However, the in-trans expression of the functional *covS* gene did not alter *emm* expression and M protein production in MGAS5005Δ*sagA* (**Fig. 3B and C**). Similarly, *emm* expression and M protein production were not altered by deletion of *covS* in MGAS2221Δ*sagA*-1 (**Fig. 3B and C**). Thus, difference in effect of *sagA* deletion on *emm* expression between MGAS2221 and MGAS5005 was not related to the functionality of the *covS* gene.

Mixed effects of *sagB* deletion on *emm* expression in MGAS2221

The conflicting results obtained for the effect of *sagA* deletion between MGAS2221 and MGAS5005 suggested that the downregulation of *emm* expression in MGAS2221Δ*sagA*-1 and MGAS2221Δ*sagA*-2 might not be related to deletion of *sagA*. If altered expression of *emm* is a common artifact of the targeted gene deletion process and independent of the specific gene being targeted, we should be able to obtain mutants with the downregulation of the M protein production when we delete genes that have been shown to have no role in *emm* regulation. We tested this hypothesis by deleting the *sagB* gene because it was shown to not alter *emm* expression in an investigation of the *sagA/pel* locus of the M1 SF370 or RND29 strain.[12] The procedure used to generate the MGAS2221Δ*sagA* mutants was followed to delete

395 bp (bases 526-to-920) of the MGAS2221 *sagB* gene, and three deletion mutants were obtained from a single attempt. One of the three mutants, MGAS2221Δ*sagB*-1, had downregulated *emm* transcription and had no detectable M protein production in western blot analysis whereas two of the three MGAS2221 *sagB* deletion mutants had normal *emm* transcription and M protein production (**Fig. 4**). These findings are consistent with our hypothesis that strains exhibiting altered *emm* expression arise as an artifact of the laboratory manipulations of the deletion process independent of the gene being deleted.

The *sagA/pel* locus in MGAS2221 does not have a role in regulating *emm* expression

The results on the effect of the *sagA* and *sagB* deletions on *emm* expression imply that the downregulation of *emm* expression in MGAS2221Δ*sagA*-1, MGAS2221Δ*sagA*-2, and MGAS2221Δ*sagB*-1 was independent of the *sagA* or *sagB* deletion. To provide supporting evidence for the contention that *sagA* does not regulate *emm* expression, we attempted to generate MGAS2221 *sagA* deletion mutants with normal *emm* expression. In our standard GAS gene deletion protocol plasmid integrants resulting from a single crossover homologous recombination were grown in THY broth for many generations to allow for a second crossover event to generate deletion mutants. We speculated that the growth in liquid medium, especially late into the stationary growth phase, may select mutants with downregulation of *emm* expression. Thus, we grew bacteria on THY agar plates for the second crossover by collecting bacteria from and plating them on agar plates every 12 h. In this way, we obtained two MGAS2221Δ*sagA* mutants. Both the mutants had wild-type *emm* transcription and M protein production (lanes or bars 6 and 7 in **Fig. 2**). These results strongly argue that the downregulated *emm* expression in MGAS2221Δ*sagA*-1 and MGAS2221Δ*sagA*-2 was not directly caused by the *sagA* deletion and that the *sagA* locus has no regulatory role in *emm* expression.

Downregulation of the M protein production in MGAS2221Δ*sagA*-1 was stable

The phase switch variants described in the studies of the Cleary and Podbielski groups have two features.[15,16] First, variants show different colony sizes from the starting strains, and, second, the variation is reversible. As shown in **Figure 5A and B**, MGAS2221Δ*sagA*-1 and MGAS2221 had similar colony size. To determine whether the downregulation of the M protein production is stable in the absence of a selection for the restoration of M protein production, MGAS2221Δ*sagA*-1 was passed on THY agar plate for 8 times, and 9 colonies were randomly chosen and analyzed for the M protein production. All the colonies still had downregulation of the M protein production (lanes 6 to 14 in **Fig. 5C**). In addition, unlike the phase switch variants in the study of Leonard et al.,[16] our deletion mutants with downregulation of the M protein production had no alteration in the SpeB production compared with the strains with normal M protein production (**Fig. 1C**). Taken together these results suggest that our deletion mutants exhibiting downregulated M-protein production differ in character from the phase switching variants described by the Cleary and Podbielski groups.

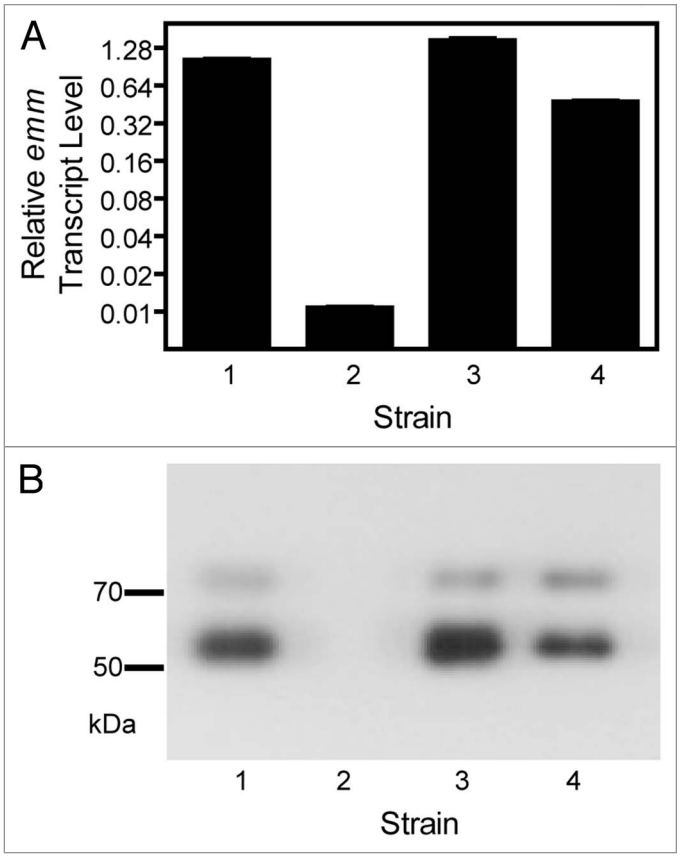

Figure 4. Mixed effects of *sagB* deletion on *emm* expression. Relative levels of *emm* transcript (**A**) and western blot detecting the production of the M protein (**B**) in the following strains: 1, MGAS2221; 2, MGAS2221Δ*sagB*-1; 3, MGAS2221Δ*sagB*-2; 4, MGAS2221Δ*sagB*-3. The Δ*sagB* mutants were obtained using THY broth for GAS growth during the gene deletion process. Levels of *emm* mRNA were normalized to that of MGAS2221.

Downregulation of the *mga* gene in the M protein expression-negative Δ*sagA* and Δ*sagB* mutants

The downregulation of the M protein production in the M protein expression-negative strains might be due to mutations in the promoter region of *emm* or *mga*. Thus, we sequenced a 449-bp fragment from upstream base −397 to base 52 in the coding region of *mga* and a 352-bp fragment covering bases −194 to +158 of *emm* for the MGAS5005, MGAS5005Δ*sagA*, MGAS2221, 4 MGAS2221Δ*sagA* mutants, and 3 MGAS2221Δ*sagB* strains. All these strains had the identical sequences in these regions. However, levels of the *mga* transcripts in the M protein expression-negative strains MGAS2221Δ*sagA*-1, MGAS2221Δ*sagA*-2, and MGAS2221Δ*sagB*-1 were 14%, 17%, and 10% of that in MGAS2221, respectively. In contrast, the M protein expression-positive Δ*sagA* and Δ*sagB* mutants had similar levels of *mga* mRNA with the parent strains (**Fig. 6**). Apparently, the M protein expression-negative phenotype of MGAS2221Δ*sagA*-1, MGAS2221Δ*sagA*-2, and MGAS2221Δ*sagB*-1 was due to the downregulation of the *mga* regulator. Unfortunately, we do not know what caused the downregulation of *mga* expression in these strains yet.

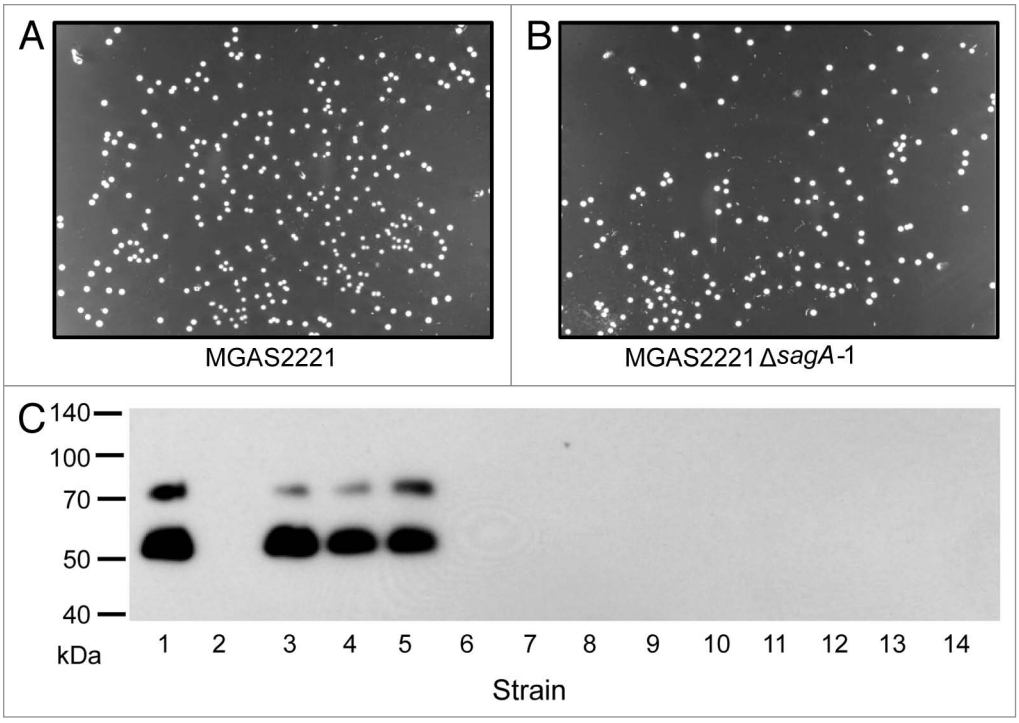

Figure 5. (**A and B**) Pictures for colonies of MGAS2221 (**A**) and its MGAS2221Δ*sagA*-1 (**B**) under the reversed background. (**C**) Western blot for detecting the M protein for the stability of the *emm*-downregulating phenotype of MGAS2221Δ*sagA*-1. The mutant and MGAS2221 were passed on THY agar plates for 8 times. After the last passage, GAS bacteria were collected from plates and plated on THY agar plate at appropriate dilution, and 3 (lanes 3–5) and 9 (lanes 6–14) colonies were randomly picked from the MGAS2221 and MGAS2221Δ*sagA*-1 samples, respectively, for western blotting analyses. Lanes 1 and 2 are the starting MGAS2221 and MGAS2221Δ*sagA*-1, respectively.

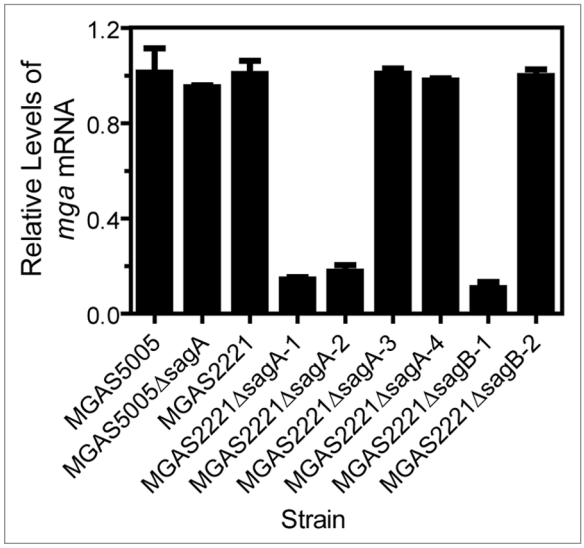

Figure 6. Relative levels of *mga* mRNA in MGAS5005, MGAS2221, and their Δ*sagA* or Δ*sagB* mutants. Levels of *mga* mRNA were normalized to that of the corresponding wild-type strain.

Discussion

This report presents findings showing that in the GAS M1T1 genetic lineage *sagA* deletion is insufficient in and of itself to reproducibly cause downregulation of M protein production. The significance of our findings is 3-fold. First, consistent with the results of Perez et al.,[14] our results demonstrate that the *sagA* locus does not regulate *emm* expression in the M1T1 lineage. Second, our results raise a concern that targeted gene deletion mutants may have downregulation of M protein production that is not directly associated with the absence of target gene, resulting in a false conclusion on the function of the target gene. Third, we provide a simple protocol to reduce the occurrence of downregulation of M protein production in the generation of GAS gene deletion mutants.

A unique observation in this study is that we obtained mixed results regarding the effect of *sagA* deletion on *emm* expression in MGAS2221. Some Δ*sagA* mutants had diminished *emm* transcription and nondetectable M protein production whereas others had normal *emm* expression and M protein production. In other words, the deletion of the *sagA* gene in a same GAS isolate obtained the results that reflect the contradicting results in a number of published studies regarding a role of the *sagA*/*pel* locus in gene regulation.[10-14] Our results suggest that the downregulation of *emm* expression described in the literature could be caused by a separate genetic event during manipulation of GAS for targeted gene inactivation.

The insertion of Tn916 at the promoter region of the *sagA* gene in a M1 isolate and a M18 strain abolished the β-hemolytic or SLS activity but had no effect on the production of the M protein.[10] Sumby and colleagues found that deletion of the *sagA*/*pel* locus caused only changes in *sagA* and *sagB* expression in M1T1 isolates, including MGAS2221, SF370, and MGAS5005.[14] We showed that MGAS2221Δ*sagA*-3, MGAS2221Δ*sagA*-4, and MGAS5005Δ*sagA* mutants had similar levels of *emm* mRNA with their parent strain according to real-time quantitative RT-PCR. Furthermore, we showed the two major bands of the M protein from the digestion of these Δ*sagA* and wild-type GAS bacteria with the phage lysin PlyC[27] in western blots. The blotting pattern is the same with that in the study of Raz et al.,[28] which used the PlyC treatment to release the M protein from the cell wall, and the M protein band with the higher molecular weight was an indication of the M protein linkage to the residual moiety of the peptidoglycan. Thus, the M protein produced

in strains of MGAS2221Δ*sagA*-3, MGAS2221ΔsagA-4, and MGAS5005Δ*sagA* was full-length and linked to the peptidoglycan. Our data supports the findings of the Sumby study[14] that the *sagA*/*pel* locus does not have a regulatory role in gene expression in the M1T1 genetic background and additionally demonstrates that it does not affect posttranscriptional processing and surface localization of intact M protein in M1T1 GAS.

Our results also suggest that the downregulation of the M protein production observed in several previous studies might be artifactual and due to spurious genetic polymorphisms other than changes in the *sagA* promoter or gene. The insertion of Tn917 transposon at the promoter region of the promoter region of *sagA* in an M49 serotype GAS strain abolished *sagA* expression and β-hemolytic activity, and this mutant also displayed diminished or reduced expression of *emm*, *speB*, and *ska* genes.[11] Thus, the *sagA* locus was given the alternate designation, Pel, for pleiotropic effect locus. However, the phenotype of this mutant strain was apparently not stable as passage of this Tn917 insertion mutant in mice selected isolates from the kidney that restored *sagA*/*pel* transcription and β-hemolytic activity but did not restore the expression of the other Tn917 insertion-affected virulence genes in the original mutant strain,[29] showing that the Tn917 insertion in the upstream of *sagA* was insufficient in and of itself to cause the pleiotropic effects. Mangold et al. proposed that the *sagA* locus contains *pel* as a small regulatory RNA.[12] The proposal that the *pel* locus functions as a regulatory RNA was based on the finding that deletion of *sagA*/*pel* (i.e., elimination of transcription was sufficient), but not eliminating *sagA*/*pel* translation (i.e., elimination of translation was insufficient), resulted in altered *emm* and sic transcription relative to the wild-type parent in the serotype M1 strain RDN29. Furthermore, the downregulation of *emm* expression in the Δ*sagA* mutant of Mangold et al. could not be repeated in the study of Sumby et al.[14] using the strain SF370 (the strain RDN29 is the strain SF370).

Genetic changes responsible for the downregulation of the M protein production in some MGAS2221Δ*sagA* mutants might be related to the variations that have been termed as phase variation.[15,16] It remains unknown whether phase variation is caused by a phase switch. It is possible that genetic changes causing the downregulation of the M protein production were selected in response to environmental stress during stationary growth phase. The viability of GAS decreases dramatically with time at the stationary phase in THY,[30] and phase variants are likely selected during stationary phase. Stationary phase mutagenesis is a common phenomenon that accelerates adaption of microbial populations under environmental stress.[17] Expression of *emm* is regulated by a positive regulator Mga, and the Mga regulon is transcribed at high levels in GAS.[3,31] The *mga* expression is downregulated in our M protein expression-negative strains. The downregulation of the Mga regulon could provide an advantage for adaptation of GAS at the stationary phase in vitro under certain condition, which could be the basis for the selection of genetic mutations that downregulate *emm* transcription during our targeted gene deletion. If this interpretation is valid, such selection pressure would be lessened when GAS grows on THY agar plates.

The downregulation of the M protein production in targeted gene deletion also happened to *sagB* deletion mutants, suggesting that GAS mutants exhibiting reduced *emm* expression can be generated by targeted gene deletion independent of the specific gene being targeted. The frequent downregulation of *emm* expression in targeted gene deletion may be linked to the high frequency of polymorphisms in GAS global regulatory genes shown in numerous publications.[18-21] The findings are consistent with the hypothesis that some of the conflicting reports in the *sagA*/*pel* literature may be explainable by spontaneous spurious mutations. In addition, independence of frequent downregulation of *emm* expression on targeted specific gene raises the concern that the phenotype of a specific gene deletion mutant may be caused by a separate genetic alteration that causes downregulation of the M protein production. This situation might have occurred in some previously reported studies. The *dltA* gene was found to be involved in the resistance of group A *Streptococcus* (GAS) to destruction by antimicrobial peptide and neutrophils and GAS invasion of epithelial cells through analyzing a *dltA* deletion mutant;[32] however, this same mutant was later found to have diminished expression of the *emm* gene.[33] The *emm* downregulation in this *dltA* deletion mutant might be caused by a secondary mutation because a *dltA* deletion mutant in the study of Cole et al.[34] apparently had normal M protein production. Cole et al. found that M protein is essential for in vivo selection of *covRS* mutations, and their Δ*dltA* mutant undergoes *covRS* mutations as its parent strain in in vivo selection. The truncation of the M protein in the *sagA* insertion mutant in the study of Biswas et al.[35] might have been caused by a mutation or deletion in the *emm* gene. The M protein is a critical GAS virulence factor, and its downregulation due to a secondary mutation could mislead conclusions as to the function of subject gene. Complementation through plasmid-mediated in trans expression is one way to ensure a correct conclusion;[23] however, plasmid-based complementation cannot perfectly restore wild-type phenotype sometimes due to the difficulty to express target gene exactly at wild-type level and the loss of the plasmid if experiments are performed in vivo. For the purpose of ruling out a secondary mutation as the cause of a phenotype, the target gene can also be knocked into its deletion mutant without introducing any foreign DNA,[22] which would avoid the drawbacks of plasmid-mediated complementation. Besides complementation, it is necessary that GAS mutants in any study should be checked to ensure the normal expression of the *emm* gene unless a gene deletion is directly linked to *emm* downregulation. High-throughput DNA sequencing can also be used to assess the information of spontaneous spurious mutations.

Fortunately we found a simple solution to reduce the occurrence of the downregulation of *emm* expression during targeted gene inactivation in GAS by passing bacteria on THY agar plates. Although the basis for the elimination of the *emm* downregulation in the plate method is not known, it is practically useful. We have generated more than 30 GAS mutants using this approach, and all of them had normal *emm* expression. Passing bacteria on agar plates is most likely a useful procedure to reduce the frequency of undesired genetic alterations in targeted gene deletion for other bacteria as well.

Materials and Methods

Bacterial strains and growth

MGAS2221 is a M1T1 GAS strain isolated from a scarlet fever patient, and M1T1 isolate MGAS5005 was isolated from an invasive case in Ontario.[23] MGAS5005 and MGAS2221 are very closely genetically related differing by less than 20 single nucleotide polymorphisms; however, the former has a null 1-bp deletion in the *covS* gene.[24] Strains were grown in Todd-Hewitt broth supplemented with 0.2% yeast extract (THY) at 37 °C in a 5% CO_2 atmosphere. THY agar was used as the solid media.

Suicide plasmid constructs for antibiotic selection marker-free deletion mutants

In the deletion of the *sagA* gene, a DNA fragment containing the whole *sagA* gene and its 24-bp upstream and 164-bp downstream (The downstream *sagB* was intact and has 62-bp upstream sequence in Δ*sagA* mutants). Upstream and downstream flanking fragments of this DNA fragment were amplified by PCR using MGAS2221 genomic DNA and primer pairs 5′-TTAGATCTGG TGATGCAAAC TCAATTG-3′/5′-TTCTCGAGGT AACTGATAAG AACGCGAG-3′ and 5′-TTCTCGAGCT AGATAGTACC TGCTAATTAC-3′/5′-TTGGATCCGA AATGTCACGA CACGAAC-3′, respectively. The two PCR fragments were sequentially cloned into suicide vector pGRV[30] at the BglII/X*ho*I and XhoI/BamHI sites, respectively. The fused PCR product was subcloned into the vector pGRV at the BglII and *BamH*I site, yielding the suicide plasmid pΔ*sagA*.

To obtain a *sagB* mutant, the upstream (f1)- and downstream (f2)-flanking fragments of an internal 395-bp fragment (bases 526 to 920) of the *sagB* gene were amplified from the MGAS2221 genomic DNA using the primer pairs 5′-GGGGACAAGT TTGTACAAAA AAGCAGGCTA GCATCTCTAT GTGATAGTG-3′(primer 1)/5′-**ATGTCTTGAC ATCAGTAGCA A**TGAAGCACA GTTTCTGAGT G-3′ (primer 2) and 5′-**TTGCTACTGA TGTCAAGACA T**TGACACTCG TAGGAACTAA G-3′ (primer 3)/5′-GGGGACCACT TTGTACAAGA AAGCTGGGTT GATAGAGAGT TGAGTCTTG-3′ (primer 4), respectively. The f1 and f2 PCR fragments were used as template to connect the two fragments in an overlay PCR using primers 1 and 4, and the 21-bp complementary tag sequences that are underlined in primers 2 and 3 bridged the f1 and f2 fragments together in the overlay PCR. The f1–f2 PCR product was cloned into pDONR221 (Life Technologies) using the BP clonase of the Gateway® cloning technology (Life Technology), yielding pDONR221-Δ*sagB*. The f1–f2 fragment was transferred into pGRV-RFA[36] in a LR clonase reaction of the Gateway cloning technology, yielding pΔ*sagB*.

Gene deletion procedures that can result in downregulation of *emm* expression

In the generation of antibiotic selection marker-free gene deletion mutants, pΔ*sagA* or pΔ*sagB* plasmid DNA was introduced into MGAS2221 or MGAS5005 using electroporation, as previously described.[30] The suicide plasmid in each experiment was inserted into the GAS chromosome through a homologous recombination event at one flanking fragment of the targeted gene. Bacteria with the first crossover were selected on THY agar plates with 10 mg/l chloramphenicol and contained one copy of full-length target gene and one copy of the shortened target gene with deletion in PCR confirmation analysis. One randomly selected colony, which had the first crossover and normal M protein production, was grown in liquid THY without chloramphenicol for ≥8 serial passages as described earlier. The culture was plated on THY agar plates after the last passage, and resultant colonies were spotted in parallel on THY agar with and without chloramphenicol to identify potential double-crossover strains cured of the suicide plasmid rendering them chloramphenicol-sensitive. These chloramphenicol-sensitive strains were analyzed by PCR to identify deletion mutants that had a shorter PCR product than the wild-type strain.

Gene deletion procedures that reduce the frequency of the downregulation of *emm* expression

The difference between the procedures that can result in and reduce the frequency of the downregulation of *emm* expression was in the passage step for the second crossover event. In the procedures to reduce the frequency of the downregulation of *emm* expression, bacteria from the first crossover step were grown on THY agar plate. At every 12 h after plating, bacteria were recovered from a whole plate, resuspended in 10 ml THY, and vortexed for 2 min. Ten microliters of the vortexed GAS suspension was plated on THY agar plate. Deletion mutants were identified as described above after ≥6 passages.

Complement of the *covS*$^{\Delta 1bp}$ pseudo gene in MGAS5005Δ*sagA*

The plasmid, pCovSC that contained the wild-type *covS* gene and was used to complement the *covS*$^{\Delta 1bp}$ pseudo gene, has been described.[23] This plasmid was introduced into MGAS5005Δ*sagA* by electroporation, and transformants were selected on THY agar plate containing 10 mg chloramphenicol/liter and was referred as MGAS5005Δ*sagA*-pCovSC. A plasmid vector control strain, MGAS5005Δ*sagA*-pDCBB, was obtained from the transformation of MGAS5005 with pDCBB.[23]

Quantitative RT-PCR analysis

Strains were grown at 37 °C, 5% CO_2, to an OD_{600} of 0.2 in THY. Total RNA was isolated from these GAS samples, as described previously.[30] High RNA quality was confirmed using an Agilent 2100 Bioanalyzer and an RNA 6000 Lab Chip kit (Agilent Technologies). TaqMan quantitative RT-PCR assays were performed using specific probes and primers (**Table 1**) and the ABI 7500 Fast System (Applied Biosystems Inc.), as described previously.[37] Control reactions that did not contain reverse transcriptase revealed no contamination of genomic DNA in any RNA sample. All RNA samples were assayed in triplicate, and the levels of *emm*, *hasA*, or *mga* mRNA were compared using the $\Delta\Delta C_T$ method with normalization to the mRNA levels of the *gyrA* gene and presented with normalization to that of corresponding wild-type or control strain.

Detection of M protein production by western blotting

Wild-type MGAS5005, MGAS2221, and their *sagA* or *sagB* deletion mutants in 10 ml cultures were harvested at an OD600 of 0.2 by centrifugation, suspended in 0.2 ml PBS were digested with 10 μg PlyC at room temperature for 1 h. Supernatant

Table 1. Oligonucleoide primers and fluorescent probes used to quantitate mRNA

Gene	Paired primers (5′-3′)	Probe[a] (5′-3′)
emm	AGACAGTTAC CATCAACAGG TGAAAC TACTCCAGCT GTTGCCATAA CAG	TAACCCATTC TTCACAGCGG CAGCC
hasA	ACCGTTCCCT TGTCAATAAA GG CGTCAGCGTC AGATCTTTCA AA	CGCCATGCTC AAGCGTGGGC
mga	GCGTTTGATA GCATCAAACA AGA CATCAAGGAG ATGAACCCAG TTG	TCACTTTTCG ACAGCCCGTT GGTGA
gyrA	CGACTTGTCT GAACGCCAAA TTATCACGTT CCAAACCAGT CAA	CGACGCAAAC GCATATCCAA AATAGCTTG

[a]Covalently linked at the 5′ end to 5-carboxylfluorescein and at the 3′ end to N,N,N-tetramethyl-6-carboxyrhodamine.

samples containing the released cell wall proteins were diluted with 1:60 1× SDS-PAGE loading buffer, and proteins in 10 μl of each sample were resolved by SDS-PAGE and analyzed by western blotting using rabbit anti-M1 protein, as described.[22] Briefly, proteins separated by SDS-PAGE were transferred to nitrocellulose membrane (Immobilon-NC, Millipore Corporation) with Towbin transfer buffer using a Trans-Blot Semi-Dry Transfer Cell (Bio-Rad Laboratories) at 15 V for 40 min. The membrane was blocked with 3% bovine serum albumin–0.1% Tween 20 in Tris-buffered saline for 1 h and incubated for 1 h with 1:4000 anti-M1 antisera. The membrane was then rinsed twice and washed three times for 15 min with 0.1% Tween 20 in PBS. The membrane was incubated with goat anti-rabbit IgG (heavy + light chain) HPR conjugate secondary antibodies for 1 h and rinsed and washed as described above. Antigen-antibody reactivity was visualized by enhanced chemiluminescence.

DNA sequencing

A DNA fragment containing the promoter of *mga* and *emm* was amplified from testing strains using the paired primers 5′-ATGGTTGTAC CATAACAGTC A-3′/5′-AGTGGAGAGA ACTAAAATTA A-3′ and 5′-CACAACTTAG ACAGCCTAAC T-3′/5′-TCCTAGGGAA GTTATAGAAG A-3′, respectively. DNA sequencing of the amplified PCR products was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit and an Applied Biosystems 3130 genetic analyzer. Sequence data were analyzed using the software Sequencer 5.1 from the Gene Codes Corporation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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