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 (MGAS2221ΔsagA) 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 ΔsagA mutant of another closely genetically related M1T1 isolate had normal *emm* expression. The *sagB* gene does not regulate *emm*; however, one of three MGAS2221ΔsagB mutants had diminished *emm* expression. The *emm* regulator *mga* was down-regulated in these M protein expression-negative strains. These results argue that *sagA* deletion does not directly cause the down-regulation of *emm* expression. Indeed, two MGAS2221ΔsagA 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 comensurately 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.1,2 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*).7 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
frequently accumulate mutations in response to nutritional stress during the stationary phase. 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. 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 MGAS2221ΔsagA 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. 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 (MGAS2221ΔsagA). One passage equaled to the growth from an optical density at 600 nm (OD600) of 0.05 to the late log growth phase with an OD600 of about 0.7 during day and the growth from OD600 of 0.05 to the late stationary growth phase with settled bacteria to the bottom on the plasmid and its homologous sequence in the chromosome, resulting in a derivative strain that was resistant to chloramphenicol. In consistent results on the effect of sagA/pel 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, MGAS5005ΔsagA; 3, MGAS2221; 4, MGAS2221ΔsagA-1; 5, MGAS2221ΔsagA-2; 6, MGAS2221ΔsagB-1; 7, MGAS2221ΔsagA-4; 8, MGAS2221; 9, MGAS2221ΔsagB-1; 10, MGAS2221ΔsagB-2; and 11, MGAS2221ΔsagB-3.

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, MGAS5005ΔsagA; 3, MGAS2221; 4, MGAS2221ΔsagA-1; 5, MGAS2221ΔsagA-2; 6, MGAS2221ΔsagB-1; 7, MGAS2221ΔsagA-4; 8, MGAS2221; 9, MGAS2221ΔsagB-1; 10, MGAS2221ΔsagB-2; and 11, MGAS2221ΔsagB-3.
Next, we measured *emm* transcription and M protein production in MGAS5005\(\Delta\)sagA\(-pCovSC\) and MGAS2221\(\Delta\)sagA\(\Delta\)covS. The introduction of the wild-type *covS* gene in MGAS5005\(\Delta\)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 MGAS5005\(\Delta\)sagA. However, the in-trans expression of the functional *covS* gene did not alter *emm* expression and M protein production in MGAS5005\(\Delta\)sagA\(\Delta\)covS (Fig. 3B and C). Similarly, *emm* expression and M protein production were not altered by deletion of *covS* in MGAS2221\(\Delta\)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 MGAS2221\(\Delta\)sagA\(-1\) and MGAS2221\(\Delta\)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. The procedure used to generate the MGAS2221\(\Delta\)sagA mutants was followed to delete...
395 bp (bases 526-to-920) of the MGAS2221 sagaB gene, and three deletion mutants were obtained from a single attempt. One of the three mutants, MGAS2221ΔsagB-1, had downregulated emm transcription and had no detectable M protein production in western blot analysis whereas two of the three MGAS2221 sagaB 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 sagaA/pel locus in MGAS2221 does not have a role in regulating emm expression

The results on the effect of the sagaA and sagaB deletions on emm expression imply that the downregulation of emm expression in MGAS2221ΔsagA-1, MGAS2221ΔsagA-2, and MGAS2221ΔsagB-1 was independent of the sagaA or sagaB deletion. To provide supporting evidence for the contention that sagaA does not regulate emm expression, we attempted to generate MGAS2221 sagaA 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 MGAS2221ΔsagA 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 MGAS2221ΔsagA-1 and MGAS2221ΔsagA-2 was not directly caused by the sagaA deletion and that the sagaA locus has no regulatory role in emm expression.

Downregulation of the M protein production in MGAS2221ΔsagA-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, MGAS2221ΔsagA-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, MGAS2221ΔsagA-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., 15 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 sagaB 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, MGas2221ΔsagB-1; 3, MGas2221ΔsagB-2; 4, MGas2221ΔsagB-3. The ΔsagB mutants were obtained using THY broth for GAS growth during the gene deletion process. Levels of emm mRNA were normalized to that of MGAS2221.](Image 312x414 to 564x732)

Downregulation of the mga gene in the M protein expression-negative ΔsagA and ΔsagB 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, MGAS5005ΔsagA, MGAS2221, 4 MGAS2221ΔsagA mutants, and 3 MGAS2221ΔsagB strains. All these strains had the identical sequences in these regions. However, levels of the mga transcripts in the M protein expression-negative strains MGAS2221ΔsagA-1, MGAS2221ΔsagA-2, and MGAS2221ΔsagB-1 were 14%, 17%, and 10% of that in MGAS2221, respectively. In contrast, the M protein expression-positive ΔsagA and ΔsagB mutants had similar levels of mga mRNA with the parent strains (Fig. 6). Apparently, the M protein expression-negative phenotype of MGAS2221ΔsagA-1, MGAS2221ΔsagA-2, and MGAS2221ΔsagB-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.
This report presents findings showing that in the GAS MIT1 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 MIT1 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 ΔsagA mutants had diminished emm transcription and non-detectable 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 MIT1 isolates, including MGAS2221, SF370, and MGAS5005.14 We showed that MGAS2221ΔsagA-3, MGAS2221ΔsagA-4, and MGAS5005ΔsagA 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 ΔsagA and wild-type GAS bacteria with the phage lysin PlyC27 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 MGAS2221ΔsagA-3, MGAS2221ΔsagA-4, and MGAS5005ΔsagA was full-length and linked to the peptidoglycan. Our data supports the findings of the Sumby study 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. 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, 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. 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 ΔsagA mutant of Mangold et al. could not be repeated in the study of Sumby et al., using the strain SF370 (the strain RDN29 is the strain SF370).

Genetic changes responsible for the downregulation of the M protein production in some MGAS2221ΔsagA mutants might be related to the variations that have been termed as phase variation. 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, and phase variants are likely selected during stationary phase. Stationary phase mutagenesis is a common phenomenon that accelerates adaptation of microbial populations under environmental stress. Expression of emm is regulated by a positive regulator Mga, and the Mga regulon is transcribed at high levels in GAS. 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. 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; however, this same mutant was later found to have diminished expression of the emm gene. 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. apparently had normal M protein production. Cole et al. found that M protein is essential for in vivo selection of covRS mutations, and their ΔdltA mutant undergoes covRS mutations as its parent strain in vivo selection. The truncation of the M protein in the sagA insertion mutant in the study of Biswas et al. 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 trans expression is one way to ensure a correct conclusion; 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, 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.23 Strains were grown in Todd-Hewitt broth supplemented with 0.2% yeast extract (THY) at 37 °C in a 5% CO2 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 ing the whole sagA gene and its 24-bp upstream and 164-bp pairs 5-bp stream 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 AAGCCGAG-3′ and 5′-TTCTCGAGGT AGATAGTACC TGCTAATTAC-3′/5′-TTGATCCGA AATGTCACGA CAGAAC-3′, respectively. The two PCR fragments were sequentially cloned into suicide vector pGRV30 at the BglIII/Xhol and Xhol/BamHI sites, respectively. The fused PCR product was subcloned into the vector pGRV at the BglIII and BamHI site, yielding the suicide plasmid pΔsagA.

To obtain a sagB mutant, the upstream (f1)- and downstream (f2)-flanking fragments of an internal 395-bp fragment were amplified by PCR using MGAS2221 genomic DNA and the primer pairs 5′-GGGGACAAGTTGTACAAAAAAGCAGGCTAGATCTCTATGTGATAGTG-3′ (primer 1)/5′-ATGTCTTGACATCAGTAGCAATGAAGCACA-TGACACTCGTAGGAACTAA (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 2, and the 21-bp complementary tag sequences to the PCORF as a primer pair, respectively. The overlay PCR product was cloned into pDONR221 (Life Technologies) using the BP clonase of the Gateway® cloning technology (Life Technology), yielding pDONR221-ΔsagB. The f1–f2 fragment was transferred into pGRV-RFA48 in a LR clonase reaction of the Gateway cloning technology, yielding pΔsagB.

Gene deletion procedures that can result in downregulation of emm expression
In the generation of antibiotic selection marker-free gene deletion mutants, pΔsagA or pΔsagB plasmid DNA was introduced into MGAS2221 or MGAS5005 using electroporation, as previously described.10 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 covSΔinp pseudo gene in MGAS5005ΔsagA
The plasmid, pCovSC that contained the wild-type covS gene and was used to complement the covSΔinp pseudo gene, has been described.23 This plasmid was introduced into MGAS5005ΔsagA by electroporation, and transformants were selected on THY agar plate containing 10 mg chloramphenicol/liter and was referred to as MGAS5005ΔsagA-pCovSC. A plasmid vector control strain, MGAS5005ΔsagA-pDCBB, was obtained from the transformation of MGAS5005 with pDCBB.23

Quantitative RT-PCR analysis
Strains were grown at 37 °C, 5% CO2, to an OD600 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 ∆∆Ct 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 GlyC at room temperature for 1 h. Supernatant

704 Virulence Volume 4 Issue 8
samples containing the released cell wall proteins were diluted with 1:60 1x 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. 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 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 using the paired primers 5′-CTTCTATGTTGAC CATAACAGTC A-3′ and 5′-ATGGTTGTAC CATAACAGTC A-3′. 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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