Natural Variation in the Promoter of the Gene Encoding the Mga Regulator Alters Host-Pathogen Interactions in Group A Streptococcus Carrier Strains

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Humans commonly carry pathogenic bacteria asymptomatically, but the molecular factors underlying microbial asymptomatic carriage are poorly understood. We previously reported that two epidemiologically unassociated serotype M3 group A Streptococcus (GAS) carrier strains had an identical 12-bp deletion in the mga promoter, a global positive gene regulator. Herein, we report on studies designed to test the hypothesis that the identified 12-bp deletion in the mga promoter alters GAS virulence, thereby potentially contributing to the asymptomatic carrier phenotype. Using allelic exchange, we introduced the variant promoter into a serotype M3 invasive strain and the wild-type promoter into an asymptomatic carrier strain. Compared to strains with the wild-type mga promoter, we discovered that strains containing the promoter with the 12-bp deletion produced significantly fewer mga and Mga-regulated gene transcripts. Consistent with decreased mga transcripts, strains containing the variant mga promoter were also significantly less virulent in in vivo and ex vivo models of GAS disease. Further, we provide evidence that the pleiotropic regulator protein CodY binds to the mga promoter and that the 12-bp deletion in the mga promoter reduces CodY-mediated mga transcription. We conclude that the naturally occurring 12-bp deletion in the mga promoter significantly alters the pathogen-host interaction of these asymptomatic carrier strains. Our findings provide new insight into the molecular basis of the carrier state of an important human pathogen.

A symptomatic carriage is a common but poorly understood phenomenon that occurs for many human bacterial pathogens, including Neisseria meningitidis (1), Streptococcus pneumoniae (2), Staphylococcus aureus (3), and Streptococcus pyogenes (group A Streptococcus [GAS]). Carriage of bacterial pathogens is clinically important because in some individuals carriage may be a prelude to invasive infection (4–11). In contrast, some early studies suggest that GAS carrier strains have reduced ability to cause disease (12–14). A combination of pathogen and host factors is believed to contribute to the development of asymptomatic carriage, but we understand little about the molecular basis by which major bacterial pathogens are able to colonize humans for prolonged periods in the absence of symptoms. The recent development of whole-genome sequencing techniques has provided an opportunity to generate novel insights into the molecular determinants of asymptomatic carriage. Importantly, in stark contrast to the analysis of the molecular genetics of virulence, little effort has been devoted to examining the bacterial determinants of asymptomatic carriage on a genome-scale level.

In terms of studying asymptomatic carriage, GAS is the prototypical model organism, as GAS is responsible for a wide range of diseases but may also be carried asymptptomatically for prolonged periods (15, 16). GAS infections vary in severity from superficial skin infections and pharyngitis to necrotizing fasciitis and toxic shock syndrome. GAS causes an estimated 600 million pharyngitis cases each year (17) which, left untreated, may lead to acute rheumatic fever. Individuals can carry GAS in the oropharynx or nasal epithelium for many months following resolution of clinical disease or may carry GAS with no history of clinical symptoms (15, 16). Depending on the population studied, GAS carriage rates range from 5 to 15% in children (18, 19), far exceeding rates of invasive disease, making asymptomatic carriage the prevailing state of GAS on human mucosal and skin surfaces. Paradoxically, few studies have investigated the contributory molecular determinants of this common phenomenon.

Although asymptomatic carriage of GAS has been studied for 60 years, the bacterial determinants remain poorly understood. Pathogenic GAS strains produce a hyaluronic acid capsule that is structurally identical to that found in human tissues and thus is not an immunological target. The immune response to GAS focuses mainly on freely secreted and cell wall-anchored proteins, such as the critical M protein that forms the basis of GAS serotyping. Serotype-specific anti-M protein antibody appears to provide protection from infection (20, 21), but reinfection with different

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A key regulatory protein contributes to the phenotypic differences.

Promoter region of GAS carrier strains reduces GAS virulence in mouse.

A putative CodY-binding site is indicated adjacent to the VNTR region. The short DNA sequence used in subsequent CodY-binding assays is indicated (black line) beneath the putative CodY-binding site.

The VNTR region residing in the P2 promoter of mga is conserved in invasive GAS strains. The 12-bp deletion in the human carrier strains MGAS12501 and MGAS12504 is highlighted in red in the invasive strain sequence. The putative CodY-binding site is indicated by a blue box.

FIG 1 The major promoter of mga differs between carrier and invasive GAS strains. (A) The P2 promoter and −10 and −35 positions (green boxes) are known (25, 26) and are indicated in relation to the mga gene start codon (black box). The deletion present in the two carrier strains is shaded and resides in a region (red boxes) between the P2 promoter start site and the beginning of the coding sequence of mga. (B) The VNTR region residing in the P2 promoter of mga is conserved in invasive GAS strains.

Mechanism contributing to altered virulence and asymptomatic colonization, a critical, but poorly understood, aspect of bacterial host-pathogen interaction.

MATERIALS AND METHODS

Bacterial strains and culture methods. The serotype M3 GAS strains used are listed in Table S1 in the supplemental material. Asymptomatic carrier strains MGAS12501 and MGAS12504 were isolated in a population-based carrier study (27) from unrelated, healthy individuals with no recent history of pharyngitis. The genomes of strain MGAS12501 and MGAS12504 have been sequenced and have a 12-bp deletion in the promoter region of mga relative to MGAS315 (22). Serotype M3 strain MGAS315 was isolated in the late 1980s from a patient in the United States with streptococcal toxic shock-like syndrome (28). Strain MGAS315 has been characterized extensively, and its genome has been sequenced (29). The other serotype M3 GAS strains used (MGAS9937, MGAS10863, MGAS10870, and MGAS15049) were cultured from patients with invasive infection in Ontario, Canada (22, 30). The genome of each of these four strains has been sequenced (30, 31). GAS strains were grown on Typicolyso agar containing 5% sheep blood agar (SBA) (Becton-Dickinson, Cockeysville, MD), in Todd-Hewitt broth containing 0.2% (wt/vol) yeast extract (THY) (Difco Laboratories, Detroit, MI), or on THY agar. When needed, GAS medium was supplemented with chloramphenicol (Sigma-Aldrich, St. Louis, MO) at 10 μg/ml. For cloning experiments, we used Escherichia coli DH5α or TOP10 (Invitrogen) grown in Luria-Bertani (LB) broth or on LB agar (Difco Laboratories) and supplemented with ampicillin (Sigma-Aldrich) at 100 μg/ml or chloramphenicol (Sigma-Aldrich) at 20 μg/ml when appropriate. The E. coli strain used for protein overexpression was grown in LB broth, and ampicillin was added to a final concentration of 80 μg/ml.

PCR and allelic exchange in strains MGAS315 and MGAS12504.

Primers for PCR and plasmids used in this study are listed in Table S2 in the supplemental material. Detailed methods regarding the generation of isolaclie mutants of the mga promoter in MGAS315 and MGAS12504 are given in the supplemental material. Briefly, the temperature-sensitive E. coli--Gram-positive shuttle vector pL1055 (32, 33) was used for allelic replacement as previously described (34). The mga promoter region and...
flanking DNA sequence were confirmed in each background using Sanger DNA sequencing as previously described (34).

**Generation of isogenic codY deletion mutant in MGAS315 and MGAS12504.** Primers used for the insertional inactivation of codY are listed in Table S2 in the supplemental material. Insertional inactivation of codY was performed as described previously (35). Briefly, a three-step process was used to generate a PCR fragment containing a spectinomycin resistance cassette (spc) with flanking DNA sequence of codY from MGAS315 or MGAS12504. The PCR fragment was subsequently cloned into a plasmid and then used for electroporation of GAS. Isogenic mutant strains were selected on medium containing spectinomycin, and the gene disruption was confirmed by DNA sequencing.

**Animal virulence experiments.** Immunocompetent female CD1 mice (Harlan Laboratories) were used for virulence studies as described previously (36). Mice were randomly assigned to treatment groups and inoculated in the right hind limb with 1 × 10^5 CFU of GAS in 100 μl of phosphate-buffered saline (PBS). Stocks of each strain were prepared at known CFU and stored at −80°C. Inocula were prepared by diluting frozen stocks in PBS to the desired number of CFU. Near mortality was determined by observation using predefined criteria (36). Mice were euthanized with isoflurane. All mouse experiments were approved by the Institutional Animal Care and Use Committee of the Houston Methodist Research Institute.

Adult cynomolgus macaques (Macaca fascicularis) (Charles River BRF) were used for the nonhuman primate experiments. All monkey experiments were performed as previously described (36). We inoculated each animal (n = 3 per strain treatment group) with 1 × 10^6 CFU/kg of strain MGAS315 or MGAS12501. Detailed information regarding experiments with nonhuman primates is given in the supplemental material. The study protocol was approved by the Institutional Animal Care and Use Committee, University of Houston.

**Growth in human blood.** Experiments assessing the ability to grow in human blood were conducted under a Houston Methodist Research Institute Institutional Review Board human subjects protocol. Experiments were carried out as described by Lancefield (37) and Flores et al. (38). A minimum of three healthy, nonimmune adult donors were used for each experiment. Briefly, cultures of strains grown overnight in THY at 37°C supplemented with 5% CO2 and were used to inoculate fresh, prewarmed THY. Cultures were grown to mid-exponential phase, and bacteria were pelleted and resuspended in an equal volume of PBS. Each strain was subsequently diluted to approximately 1 × 10^5 CFU from which 10 to 100 CFU of GAS was used to inoculate 300 ml of fresh human blood. Samples were incubated at 37°C with 5% CO2 with gentle rotation for 3 h and then serially diluted in PBS and immediately plated on SBA for CFU. The multiplication factor was calculated by dividing the resulting CFU/ml after 3 h of incubation by the starting inoculum.

**RNA isolation and quantitative real-time PCR analysis.** To analyze mga transcript over time, GAS strains were grown overnight in THY, diluted 1:100 in fresh THY, and incubated. Samples were taken hourly for determination of optical density at 600 nm (OD600) and RNA isolation and purification beginning 2 h postdilution (early exponential phase) for up to 10 h (stationary phase). RNA was isolated and purified using an RNeasy 96 kit according to the manufacturer’s instructions (Qiagen). The quantity and quality of RNA were determined with an Agilent 2100 Bioanalyzer. Reverse transcription of RNA to produce cDNA was done with a high-capacity cDNA reverse transcription kit (Applied Biosystems).

TaqMan quantitative real-time PCR (qRT-PCR) was performed with an ABI 7500 Fast Real-Time system, and proS (39) or tufA (40) was used as the endogenous control gene. TaqMan primers and probes used in analyses are listed in Table S2 in the supplemental material. To compare mga expression in asymptomatic carrier strains with invasive strains, the threshold cycle (ΔΔCt) method was used (user bulletin no. 2, ABI Prism 7700 Sequence Detection System; Applied Biosystems). All reactions were performed in quadruplicate using RNA purified from at least two biologic replicates.

Purification of CodY from serotype M3 GAS and EMSA. Details of the recombinant CodY purification and electrophoretic mobility shift assays (EMSA) are given in the supplemental material. Briefly, the coding region of codY was amplified from strain MGAS315 using the primers listed in Table S2 and was cloned into plasmid pET21b. Oligonucleotides used to generate oligoduplexes for EMSA were p CodY (5’-TGGAGGAA TTATCAGAAAAATTCTATT-3’), pmgA (5’-TCTTTTTTATTTTCTT GATTTTTTGTATTA-3’), and DNA sequence lacking a CodY operator (5’-ACAGACAGTGTGTTGCCACACACACTCA-3’). Binding reactions were performed at 37°C for 15 min in a 20-μl reaction volume with 0.2 μM oligoduplex and increasing concentrations of CodY.

**Western immunoblot analysis for M protein in MGAS12504 and MGAS12504mga**. GAS strains were grown in THY and cells harvested in mid-exponential phase. Cell wall extracts were obtained as previously described (41). Strain MGAS12502 was used as a negative control, as it harbors a 195-bp deletion in the emm gene deleting the hypervariable N terminus (22). Equal amounts of total protein were resolved on a 12% SDS-PAGE gel, transferred to a nitrocellulose membrane, and probed with a previously described polyclonal anti-M protein (31). M protein was detected by secondary antibody conjugated with horseradish peroxidase and visualized by chemiluminescence using Pierce ECL Western blotting substrate (Thermo Scientific).

**Statistics.** Kaplan-Meier analysis was performed to determine statistical significance of mortality data. A two-tailed t test was used to compare transcript levels at individual time points and the multiplication factors between strains grown in human blood. A two-tailed Mann-Whitney test was used to compare pathology scores for nonhuman primate virulence studies. A P value less than 0.05 was considered significant for all statistical tests.

**RESULTS**

Asymptomatic carrier strains MGAS12501 and MGAS12504 are less virulent in mouse and nonhuman primate models of necrotizing fasciitis. We previously reported that the serotype M3 asymptomatic carrier strains MGAS12501 and MGAS12504 had a significantly higher 90% near-lethal dose (LD90) (i.e., were less virulent) for mice than did invasive serotype M3 strains after intraperitoneal (i.p.) inoculation (22). To study virulence attributes of the asymptomatic carrier strains in more detail, we tested the hypothesis that strains MGAS12501 and MGAS12504 are less virulent than the invasive strain MGAS315 in a mouse model of necrotizing fasciitis (36). To compare pathology scores for nonhuman primate virulence studies. A P value less than 0.05 was considered significant for all statistical tests.

Asymptomatic carrier strains MGAS12501 and MGAS12504 caused significantly less tissue pathology than invasive strain MGAS315 (Fig. 2A). Similarly, carrier strains MGAS12501 and MGAS12504 were significantly less virulent in the mouse necrotizing fasciitis model (Fig. 2A). The carrier strains produced significantly less tissue destruction and abscess formation compared to the extensive necrosis observed in mice inoculated with invasive strain MGAS315 (Fig. 2B to D). Among the animals infected with the asymptomatic carrier strains, only two mice had a near-death phenotype, whereas all of the mice inoculated with invasive strain MGAS315 had this phenotype.

GAS is a human-specific pathogen, and several key virulence factors lack or have poor activity against mouse molecules. For example, the critical GAS virulence factor streptokinase is active in humans and nonhuman primates but not in mice (42, 43). Thus, we next sought to test the hypothesis that the asymptomatic carrier strain MGAS12501 is less virulent in a nonhuman primate model of necrotizing fasciitis (36). The use of strain MGAS12501 (versus MGAS12504) in this analysis was an arbitrary choice, as these two organisms are virtually identical (22). Consistent with...
the mouse data, strain MGAS12501 was significantly less virulent in the nonhuman primate than the invasive strain MGAS315, as assessed by lesion volume (Fig. 3A), pathology score (Fig. 3B), and magnitude and character of tissue destruction (Fig. 3C to H). Together, the mouse and nonhuman primate virulence studies clearly demonstrate a decreased-virulence phenotype in the asymptomatic carrier strains relative to the invasive strain MGAS315.

Asymptomatic carrier strains MGAS12501 and MGAS12504 have significantly decreased mga transcript levels compared to those of invasive strains. The 12-bp deletion identified in the carrier strains MGAS12501 and MGAS12504 was not present in any of the genomes of 95 invasive serotype M3 GAS strains recently studied (30), suggesting that it is unique to asymptomatic carrier strains. Further, the finding that the asymptomatic carrier strains had a 12-bp deletion in the mga promoter suggests that the carrier strains may have altered mga expression compared to that of invasive strains. Given that mga expression is growth phase dependent (44), we tested the hypothesis of decreased mga expression using quantitative real-time reverse transcription-PCR (RT-PCR) with RNA isolated over the entire GAS growth cycle. The mga transcript level increased substantially in invasive strain MGAS315 beginning at the transition from exponential to stationary growth phase and extending into stationary phase (Fig. 4A). In striking contrast, the level of mga transcript did not change significantly in the two asymptomatic carrier strains. Rather, beginning at 7 h (transition to stationary phase), we found between 2- and 22-fold less mga transcript in the asymptomatic carrier strains than in MGAS315 (Fig. 4A and B). To test the hypothesis that the significant decrease in mga transcript level observed in MGAS12501 and MGAS12504 was unique to the carrier strains, we analyzed mga transcript level in four additional serotype M3 invasive strains, all of which lack the 12-bp promoter deletion in mga (see Table S1 and Fig. S1 in the supplemental material). The asymptomatic carrier strains also had significantly lower levels of mga transcript than did these other invasive strains (Fig. S1). Finally, to ensure that the differences in mga transcript between the asymptomatic carrier and invasive strains were not an artifact caused by decreased expression of the endogenous control gene (proS) in stationary phase, we repeated the experiment with a second endogenous control gene, tufA (40). The results confirmed the finding of decreased mga transcript levels in the carrier strains (see Fig. S2 in the supplemental material).

Having established that mga expression is significantly decreased in the growth cycle of the two asymptomatic carrier strains with the 12-bp deletion in the mga promoter, we next tested the hypothesis that these strains had decreased expression of Mga-regulated genes. Thus, we determined the transcript levels of genes known to be directly regulated by Mga, including emm, scpA (encoding C5a peptidase), and sclA (encoding streptococcal collagen-like protein A). The C5a peptidase of GAS cleaves C5a, thereby inhibiting recruitment of phagocytic cells to the site of infection (45, 46), whereas SclA is a cell surface protein involved in adherence and internalization (47). Consistent with our hypothesis, the transcripts of all Mga-regulated genes tested (emm, scpA, and sclA) were significantly decreased in strains MGAS12501 and MGAS12504 compared to MGAS315 (Fig. 4B). Further, consistent with peak mga expression in MGAS315 (Fig. 4A), the greatest difference in emm transcript level was observed in the transition and stationary phases of growth (Fig. 4B). The same decreased gene transcript pattern was observed for scpA and sclA (Fig. 4B). However, differences between the scpA and sclA transcripts levels were far greater than observed for emm. It is possible that the observed differences in transcript reflect differences in Mga promoter affinity between the three genes. Our findings confirm that the transcript levels of Mga-regulated genes were decreased in the carrier isolates compared to invasive strains.

Allelic exchange demonstrates that the 12-bp deletion in mga contributes to decreased production of mga transcript and decreased virulence. Given that the carrier strains and invasive strains have modest genetic differences in addition to the 12-bp mga promoter deletion, the data presented to date do not definitively demonstrate that the 12-bp mga promoter deletion is responsible for the observed differences in virulence phenotype or transcript level. Therefore, we next directly tested the hypothesis that the 12-bp deletion in the promoter of mga in the asymptomatic carrier strains results in the observed differences between the carrier and invasive strains. We used allelic replacement (34) to introduce the 12-bp deletion into strain MGAS315 to create a

**FIG 2** Asymptomatic carrier strains are significantly less virulent in a necrotizing fasciitis model of GAS infection. (A) Survival curve of mice infected with carrier strains MGAS12501 and MGAS12504 and invasive strain MGAS315. Ten mice in each group were infected intramuscularly with 1 × 10⁴ CFU, as described in Materials and Methods. P values are relative to MGAS315 (P < 0.05) as determined by log rank test. (B to D) Gross pathology (original magnification, ×4) at 48 h and (E to G) histopathology (original magnification, ×20) of mouse hind limb lesions at 24 h postinfection. Mice infected with strain MGAS315 had extensive spreading myonecrosis and tissue destruction (B and E). In contrast, the lesions in mice challenged with the asymptomatic carrier strain MGAS12501 (C and F) or MGAS12504 (D and G) had small abscesses confined to the inoculation site. The boxed areas and arrows demarcate a circumscribed, walled-off lesion.
the hypothesis, strain MGAS315Δ12bp had a significantly decreased mga transcript level compared to that of the parental strain MGAS315 (Fig. 5A).

Given the significant differences in the transcript levels of mga, we hypothesized that the introduction of the 12-bp mga promoter deletion would result in significant virulence differences. As predicted, strain MGAS315Δ12bp was significantly attenuated compared to parental strain MGAS315 following intramuscular inoculation into mice (Fig. 5B). Compared to the invasive strain MGAS315, the isoallelic mutant strain MGAS315Δ12bp caused significantly less tissue destruction (Fig. 5C). Furthermore, in contrast to strain MGAS315, histopathologic analysis demonstrated that the surrounding muscle and soft tissue in the mice infected with the isoallelic mutant strain retained viability (Fig. 5D).

As mentioned previously, GAS is a human-specific pathogen, and the possibility exists that the decreased virulence observed in the isoallelic mutant may not exist in a model more closely resembling infection in humans. Growth in human blood is often used as a proxy for resistance to phagocytosis (48). Further, given that the isoallelic mutant has a significantly reduced mga transcript and Mga is known to positively regulate multiple virulence factors necessary for immune evasion (23, 24), we tested the hypothesis that the isoallelic mutant MGAS315Δ12bp would have significantly reduced ability to survive and grow in human blood. We observed a >2-fold reduction (P = 0.01) in the ability of MGAS315Δ12bp to grow in human blood compared to that of the parental strain (Fig. 5E). Combined with the mouse virulence data, these data further demonstrate that the 12-bp deletion in the promoter of mga contributes to decreased virulence.

Repair of the 12-bp deletion in an asymptomatic carrier strain results in increased mga and Mga-regulated gene transcript levels and increased ability to grow in human blood. As a means to confirm the finding that the 12-bp deletion in the promoter of mga contributes to decreased virulence in an invasive strain of GAS, we next tested the hypothesis that repair of the 12-bp deletion in an asymptomatic carrier strain would result in an increase in mga and Mga-regulated gene transcripts. Using allelic exchange (34), we constructed a strain that differed from the asymptomatic carrier strain MGAS12504 by only the absence of the 12-bp deletion in the promoter of mga (i.e., a wild-type mga promoter). The isoallelic mutant MGAS12504mga<sup>wt</sup> was similar in growth and hyaluronic acid capsule production to the parental strain MGAS12504. Consistent with our hypothesis, we found that the repaired carrier strain, MGAS12504mga<sup>wt</sup>, produced significantly greater mga and Mga-regulated gene transcript levels than did the parental asymptomatic carrier strain (Fig. 6A). Consistent with increased emm transcript, we detected an increase in M protein in MGAS12504mga<sup>wt</sup> using antibody specific for M protein in serotype M3 GAS (Fig. 6B).

We hypothesized that similar to the isoallelic mutant MGAS315Δ12bp, the carrier strain harboring the 12-bp deletion in the promoter of mga would have a decreased ability to evade phagocytosis and thus grow in human blood compared to that of the “repaired” carrier strain containing the wild-type mga promoter. Consistent with our hypothesis, we observed a >6-fold increase (P = 0.01) in the ability of MGAS12504mga<sup>wt</sup> to grow in human blood compared to that of the parental strain (Fig. 6C). Together, our data unambiguously demonstrate that the 12-bp

strain (MGAS315Δ12bp) that differs from its parental strain by only the 12-bp deletion. The isoallelic mutant MGAS315Δ12bp was similar in growth and hyaluronic acid capsule production to the parental strain MGAS315 (data not shown). Consistent with
deletion contributes to decreased virulence in both invasive and asymptomatic carrier strains.

**Decreased mga transcript level with variant mga promoter mediated by CodY.** It is possible that the 12-bp deletion in the asymptomatic carrier strains alters, either directly or indirectly, binding of a transcription factor, accounting for the observed mga transcript differences. Mga is known to self-regulate through binding to its own promoter (25, 26); however, the previously identified Mga binding sites are distant from the VNTR in which the 12-bp deletion occurs in the asymptomatic carrier strains (25, 26). Thus, we interrogated the VNTR and immediately adjacent nucleic acid sequence for putative transcription factor binding sites and discovered a putative binding site for the transcription factor CodY (Fig. 1A). CodY is well described for a diverse array of Gram-positive bacteria in which it is known to repress synthesis and transport of branched-chain amino acids (49). Studies of CodY in GAS have shown a similar metabolic repression (50–52) but suggest simultaneous activation of multiple virulence genes and virulence gene regulators, including mga (50, 51). However, no studies have shown direct activation of mga through CodY. We hypothesized that the 12-bp deletion in the mga promoter reduced or eliminated the ability of CodY to increase mga transcript levels. To begin to show CodY regulation of mga transcript levels, we generated isogenic mutants lacking codY in the invasive strain MGAS315 and the asymptomatic carrier strain MGAS12504. If our hypothesis is correct, mga transcript levels will be similar between MGAS315ΔcodY and MGAS315Δ12bp. Likewise, elimination of CodY in the carrier strain MGAS12504, which harbors the 12-bp deletion in the mga promoter, should have a minimal effect on mga transcription. Consistent with our hypothesis, we observed significantly decreased mga transcript in MGAS315ΔcodY compared to the wild-type parental strain, and the observed decrease was similar to that in the isoallelic mutant MGAS315Δ12bp (Fig. 7A). Also, we observed no significant decrease in mga transcript in MGAS12504ΔcodY compared to the parental strain (Fig. 7A).

The observed decrease in mga transcripts in strains lacking CodY may be an indirect effect mediated through effects on other regulators (50, 51). Thus, we next hypothesized that CodY binds to the mga promoter in a specific manner. CodY protein was purified and used in electrophoretic mobility shift assays (EMSA) with a short, double-stranded DNA oligomer representing the putative CodY binding site in the mga promoter (Fig. 1). CodY has previously been shown to bind specifically to its own promoter in serotype M49 GAS (50). As shown in Fig. 7B, CodY binds to its own promoter in serotype M3 GAS, and consistent with our hypothesis, CodY binds to the mga promoter. Importantly, while a reduction in the amount of free DNA is observed in the negative control, there is an absence of a distinct shift as observed in pcodY and pmga (Fig. 7B). Together, these data demonstrate that CodY binds to the mga promoter and increases mga transcription, and they suggest that mutations in or around the consensus CodY binding site affect regulation of mga through CodY.

**DISCUSSION**

Asymptomatic carriage is a common state of many important bacterial human pathogens, including GAS. For decades, observations have been made regarding the carrier state of these and other pathogens, but we are in our infancy in understanding the molecular factors contributing to asymptomatic bacterial carriage. Studies to date examining asymptomatic carriage of bacterial pathogens have focused on broad genetic characteristics such as the presence or absence of particular genes and epidemiologic comparisons between infecting and colonizing strains (53–56).
The data provided herein shed new light on the carrier state of pathogenic bacteria by demonstrating that a naturally occurring mutation in the promoter region of a virulence gene regulator in human carrier strains significantly alters host-pathogen interaction. Importantly, this seemingly minor genetic variation would not have been discovered with any of the typical tools currently used to analyze carrier strains such as PCR to determine gene content or multilocus sequence typing to estimate phylogenetic relationships.

The fact that the identical 12-bp deletion in the promoter of mga has arisen independently in two GAS carrier strains suggests that this genetic change provides an evolutionary advantage for the asymptomatic carrier state. Furthermore, while it does not replace the relative lack of asymptomatic carrier DNA sequences available for comparison, the fact that the 12-bp deletion does not occur in the published invasive serotype M3 genomes (22, 30, 31) suggests that the polymorphism is unique to the carrier strains examined and may confer an advantage on the asymptomatic carriers. Given the transcriptional regulation role of Mga, it is likely that alterations in the protein levels of genes regulated by Mga, such as M protein, are contributors to this process. As M protein is known to induce a strong inflammatory response (20), downregulation of M protein levels might be expected to provide a selective advantage by minimizing the immunogenicity of GAS strains in the oropharynx. As previously noted, other GAS carrier strains have been found to have decreased M protein levels (13, 14). Moreover, a study of experimental GAS pharyngitis in non-human primates found that the emm gene was downregulated during the asymptomatic carrier phase of the study (40). Further, Beres et al. (22) reported that an asymptomatic carrier strain contained a large deletion in the hypervariable region of the M protein. Given that Mga directly interacts with approximately 10% of genes in the GAS genome (57), it is possible that additional genes regulated by Mga contribute to the virulence differences we observed in strains with differing promoter structures.

The alteration in the mga promoter of the asymptomatic carrier strains in this study is due to differences in the number of variable-number tandem repeats (VNTRs) (Fig. 1). Because of their structure, VNTRs are prone to DNA replication errors resulting in high rates of either VNTR extension or deletion. In prokaryotes, VNTRs in genes encoding many cell surface molecules have been recognized as contributing to antigenic variation.
through their effects on shifting translation reading frames or altering promoter activity (58). The molecular mechanisms by which alterations in VNTRs affect promoter activity are not entirely clear, but the alterations may occur by creation or elimination of binding sites for transcription factors (59). The VNTR in the mga promoter is about 20 bp proximal to the major mga transcriptional site (25, 26), and based on the current data (Fig. 7), it appears that a larger mga promoter VNTR region allows for greater activity through the binding of CodY. The exact mechanism, e.g., activation by repressor modulation or assisting in RNA polymerase promoter escape, leading to the observed effect of CodY is unknown but under further investigation. Regardless of the mechanism by which the VNTR region affects transcription, inherent instability of the mga promoter VNTR region may allow for modulation of GAS virulence in response to diverse environmental pressures encountered by GAS during various phases of infection. Our finding of a VNTR that affects transcript of a global regulator, rather than a cell surface protein, adds to understanding of the molecular mechanisms by which pathogenic bacteria produce variation in virulence phenotype. Further, the VNTR in the promoter of mga, a well-known virulence regulator of GAS, and its effect on activation through CodY had not been previously described. Thus, our finding that the VNTR affects transcription and virulence provides a new mechanism of virulence control through mga.

Our data add to existing studies that suggest that increased virulence is not always evolutionarily beneficial to microbes. For example, our finding that GAS carrier strains downregulate virulence factor production is similar to the finding that Pseudomonas aeruginosa strains chronically isolated from a patient with cystic fibrosis had multiple mutations in virulence factor and virulence factor regulator-encoding genes (60). Further, while our studies demonstrate that the 12-bp deletion in the promoter of mga contributes to decreased virulence, it is likely that additional factors are required to complete the asymptomatic carrier phenotype. That is, multiple mutations, as observed in the adaptation of Pseudomonas aeruginosa (60, 61) and Burkholderia dolosa (62) to the airways of cystic fibrosis patients, may be required for the full asymptomatic carrier phenotype representing an adaptation to growth in a human host. In vivo studies are needed with GAS and other important human pathogens to determine the extent that genetic variation contributes to the development of asymptomatic carriage. Such carriage studies challenge the paradigm that only the investigation of virulence leads to new insights in bacterial pathogenesis.

Our findings have several implications. First, the decreased expression of emm in the asymptomatic carrier strains suggests that an M protein-based vaccine strategy may be suboptimal in eradicating the GAS carrier state given that such strains may express M protein at relatively low levels. Second, our genome-wide approach for evaluating the molecular basis of bacterial carriage highlights the importance of whole-genome sequencing of clinical isolates other than those causing severe invasive infections, as has been the typical approach for more than a decade. Similar approaches could be taken with other critical bacterial pathogens that are often carried asymptomatically such as S. aureus, S. pneumoniae, and N. meningitidis. Finally, our data demonstrate the importance of closely examining variation in noncoding regions when evaluating whole-genome sequencing data. The 12-bp deletion studied in this work is not located in a virulence factor-encoding gene or even in an open reading frame, yet it had a significant effect on GAS virulence.

The past several decades have seen dramatic advances in understanding how bacterial pathogens cause a diverse array of disease in humans. In contrast, little attention has been given to the study of molecular factors contributing to asymptomatic carriage, despite the fact that it is common for many bacterial pathogens. Our data provide new information about the GAS asymptomatic
Carry phenotype and strongly suggest that subtle genetic variation is a critical contributing factor. Further genome-wide investigation using human carrier isolates may provide additional insights into the molecular events underlying the evolution of major bacterial pathogens.

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