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Asymptomatic Carriage of Group A *Streptococcus* Is Associated with Elimination of Capsule Production

Anthony R. Flores,[a,b] Brittany E. Jewell,[a,b] Randall J. Olsen,[b] Samuel A. Shelburne III,[c] Nahuel Fittipaldi,[b]* Stephen B. Beres,[b] James M. Musser[b]

Section of Infectious Diseases, Department of Pediatrics, Texas Children's Hospital and Baylor College of Medicine, Houston, Texas, USA[a]; Center for Molecular and Translational Human Infectious Diseases Research, Houston Methodist Research Institute, Department of Pathology and Genomic Medicine, Houston Methodist, Houston, Texas, USA[b]; Department of Infectious Diseases, Division of Internal Medicine, MD Anderson Cancer Center, Houston, Texas, USA[c]

Humans commonly carry pathogenic bacteria asymptomatically, but despite decades of study, the underlying molecular contributors remain poorly understood. Here, we show that a group A streptococcus carriage strain contains a frameshift mutation in the *hasA* gene resulting in loss of hyaluronic acid capsule biosynthesis. This mutation was repaired by allelic replacement, resulting in restoration of capsule production in the isogenic derivative strain. The "repaired" isogenic strain was significantly more virulent than the carriage strain in a mouse model of necrotizing fasciitis and had enhanced growth *ex vivo* in human blood. Importantly, the repaired isogenic strain colonized the mouse oropharynx with significantly greater bacterial burden and had significantly reduced ability to internalize into cultured epithelial cells than the acapsular carriage strain. We conducted full-genome sequencing of 81 strains cultured serially from 19 epidemiologically unrelated human subjects and discovered the common theme that mutations negatively affecting capsule biosynthesis arise *in vivo* in the *has* operon. The significantly decreased capsule production is a key factor contributing to the molecular détente between pathogen and host. Our discoveries suggest a general model for bacterial pathogens in which mutations that downregulate or ablate virulence factor production contribute to carriage.

Asymptomatic carriage is a common but poorly understood phenomenon that occurs for many human bacterial pathogens, including *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Streptococcus pyogenes* (group A streptococcus [GAS]) (1–4). The fact of the matter is that these and other frank pathogens spend the majority of their life cycle peacefully coexisting with their host, rarely causing clinical symptoms. The underlying molecular mechanisms favoring carriage over disease are unknown, but a combination of pathogen and host factors is believed to be involved. However, in spite of a relatively sophisticated understanding of many bacterial pathogenesis processes, we understand little about the molecular basis by which major bacterial pathogens are able to colonize humans asymptomatically for prolonged periods.

GAS is an ideal model organism to study asymptomatic carriage mechanisms for several reasons. This organism is responsible for a variety of human infections, ranging from the severe necrotizing fasciitis and streptococcal toxic shock syndrome to the relatively benign impetigo and pharyngitis. However, in most individuals, GAS primarily exists as a naso- and oropharyngeal mucosal colonizer. Individuals can carry GAS in the oropharynx or nose for many months following resolution of clinical disease or may carry GAS asymptomatically with no antecedent history of clinical symptoms (5). Depending on the population studied, GAS carriage rates range from 5 to 15% in children (3), a rate far exceeding that of any disease caused by GAS. For example, rates of invasive infection in well-studied populations generally range from 1 to 3 cases per 100,000 (6). Thus, asymptomatic carriage represents the numerically dominant state of interaction with the human host, but little work has investigated the molecular mechanisms contributing to this common phenomenon.

Very early studies found that GAS strains isolated during carriage produce less hyaluronic acid capsule than strains isolated from infection sites (7). Strains recovered during carriage also have other decreased phenotypes, such as M protein production, hemolysis, and dissemination to other individuals (8–10). Unencapsulated strains of *N. meningitidis* have been isolated from carriers (11), suggesting that lack of capsule production is linked to carriage. Recently, a comparative genomic analysis showed that hyaluronic acid capsule biosynthesis in GAS is under strong selective pressure depending on the host environment (12). In that study, strains sequentially isolated from nonhuman primates contained mutations that negatively affected capsule production. However, the contribution and molecular underpinnings of decreased capsule production in carriage of GAS in humans remain undefined.

In a study designed to provide new molecular information about GAS carriage, Beres et al. (13) performed genome-wide polymorphism identification of four epidemiologically independent carriage strains and studied their virulence in an animal model of invasive GAS infection. Compared to strains cultured from patients with invasive infections, GAS carriage strains were significantly less virulent for mice as assessed by intraperitoneal

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Address correspondence to Anthony R. Flores, arflores@bcm.edu.
* Present address: Nahuel Fittipaldi, Public Health Ontario and Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto, Toronto, Canada.

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inoculation. Genome sequencing identified several candidate polymorphisms that may contribute to the phenotypes of decreased virulence and carriage. However, definitive studies demonstrating a causal role of these and other genetic polymorphisms to carriage have not been conducted.

Here, we report the results of studies designed to test the hypothesis that small genetic changes contribute to the carrier phenotype and decreased virulence. We discovered a single-nucleotide insertion mutation in a carriage strain that eliminated hyaluronic acid capsule production. Our functional analyses conclusively show that mutations negatively affecting capsule synthesis contribute to the phenotypic differences observed between carriage and invasive strains. Using whole-genome sequencing of 81 additional GAS strains serially cultured from 19 subjects with persistent carriage, we also demonstrate that mutations that abrogate capsule synthesis arise in the human upper respiratory tract. This common theme provides new information about the molecular basis of asymptomatic colonization, a critical but very poorly understood aspect of bacterial host-pathogen interaction.

MATERIALS AND METHODS

Bacterial strains. Carriage strain MGAS12503 was isolated in a population-based study (14) from a healthy individual with no recent history of pharyngitis; its genome has been sequenced (13). Serotype M3 strain MGAS315 was isolated in the late 1980s from a patient with streptococcal toxic shock-like syndrome (15), and the complete genome sequence is available (GenBank accession number NC_004070). Bacteria were grown on Trypticase soy agar containing 5% sheep blood agar (SBA) (Becton, Dickinson, Cockeysville, MD), in Todd-Hewitt broth containing 0.2% (wt/vol) yeast extract (THY) (Becton, Dickinson), 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 (Becton, Dickinson) and supplemented with ampicillin (Sigma-Aldrich) at 100 μg/ml or chloramphenicol (Sigma-Aldrich) at 20 μg/ml when appropriate.

Allelic exchange in carriage strain MGAS12503. Temperature-sensitive *E. coli* Gram-positive shuttle vector pJL1055 was used for allelic replacement (16, 17). To repair *hasA* in MGAS12503, a 1.5-kb fragment containing the wild-type *hasA* from MGAS315 was amplified using primer pair MSP107 (5′-TAATCTATTAACGCGACTTA-3′) and MSP114 (5′-TGGGTTATTATAATGCATTC-3′), cloned into pCR2.1-TOPO (Invitrogen) to generate pJSF21, excised using BamHI and XhoI, and ligated into the same sites of pJL1055 to generate pJSF26. Electrocompetent cells of the appropriate GAS strains were transformed with pJSF6 and allelic replacement carried out as previously described (18). Isoallelic "repaired" mutants were confirmed using DNA cycle sequencing (BigDye Terminator v3.1 cycle sequencing kit; Applied Biosystems).

Animal virulence experiments. Immunocompetent female CD1 mice (Harlan Laboratories) were used for virulence studies (19). Mice were randomly assigned to treatment groups and inoculated in the right hind limb with 1×10^7 CFU of GAS in 100 μl phosphate-buffered saline (PBS). Near-mortality was determined by observation using predefined criteria (19). 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 (19). Each animal was inoculated ($n = 3$ per strain treatment group) with 1×10^8 CFU/kg of body weight of either strain MGAS315 or MGAS12503. Each animal was anesthetized, outfitted with a transdermal fentanyl patch, and inoculated intramuscularly in the anterior thigh to a uniform depth. Animals were observed continuously, sacrificed in matched pairs, and necropsied. Infected tissue collected at necropsy was examined by one or more pathologists and veterinarians. Tissue was fixed in 10% phosphate-buffered formalin, serially sectioned, and embedded in paraffin using automated standard instruments. Microscopic pathology was scored in a blinded fashion by a pathologist using a previously validated scoring system for necrotizing fasciitis (NF) in cynomolgus macaques (19). Hematoxylin and eosin-stained and Gram-stained sections were examined with a BX5 microscope and photographed with a DP70 camera (Olympus). The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Houston.

Mouse nasopharyngeal colonization. Experiments involving mouse colonization were approved by the Institutional Animal Care and Use Committee of the Houston Methodist Research Institute and were carried out as previously described (20, 21). Female CD1 mice (Harlan Laboratories) were inoculated intranasally with either 5×10^7 CFU (MGAS12503 or MGAS12503*hasA*^{wt}) or 1×10^6 CFU (MGAS315) in 50 μl PBS. Mouse throats were swabbed prior to inoculation, to document absence of beta-hemolytic bacteria, and daily thereafter. Bacteria were released from swabs by suspending them in 300 μl PBS, serial dilutions were performed for quantitative CFU, and bacteria were plated on SBA. Beta-hemolytic colonies were counted after overnight incubation at 37°C with 5% CO_2 and tested for the presence of GAS carbohydrate antigen using latex agglutination (BD Biosciences).

Human epithelial cell internalization assays. GAS internalization to cultured epithelial cells was carried out as previously described by LaPenta et al. (22). Briefly, HaCaT cells were seeded at a density of 6×10^5 in 2 ml high-glucose Dulbecco's modified Eagle's medium (DMEM) with L-glutamine and 10% fetal bovine serum (FBS) in a 12-well plate. Cells were incubated in a dedicated incubator overnight at 38.5°C. Indicated GAS strains were grown to mid-exponential phase in THY, washed with sterile PBS, and resuspended in an equal volume of PBS. Approximately 1×10^7 CFU GAS was added to 8 replicate wells, rocked briefly, and then incubated for 1 h at 37°C. Each well was then washed twice with 1 ml PBS, twice with 2 ml PBS, and once with 1 ml PBS. Gentamicin was then added to each well at a concentration of 100 μg/ml and incubated for 1 h at 37°C to kill extracellular GAS. Each well was then washed 3 times with 1 ml PBS to remove antibiotics. HaCaT cells were released from the wells using 0.25% trypsin-1 M EDTA. Cells were lysed using sterile water, serially diluted, and plated on SBA to enumerate GAS. Percentage adherence was calculated by dividing the recovered CFU by the original inoculum.

Growth in human blood. Experiments assessing the ability of GAS to grow in human blood were conducted under a human subject protocol approved by Houston Methodist Research Institute Institutional Review Board. Experiments were carried out as described by Lancefield (23). A minimum of three healthy, nonimmune, adult donors were used for each experiment. Briefly, cultures of strains were grown overnight in THY at 37°C supplemented with 5% CO_2 and were used to inoculate fresh, prewarmed THY. Cultures were grown to mid-exponential phase, and bacteria were pelleted and suspended in an equal volume of PBS. Each strain was subsequently diluted to approximately 1×10^3 CFU from which 10 to 100 CFU of GAS was used to inoculate 300 μl of fresh human blood. Samples were incubated at 37°C with 5% CO_2 with gentle rotation for 3 h and then serially diluted in PBS and immediately plated on SBA for CFU enumeration. The multiplication factor was calculated by dividing the resulting CFU/ml after 3 h of incubation by the starting inoculum.

Genome sequencing of sequentially cultured strains. Strains were isolated from individuals enrolled in a study to investigate the human immune response to GAS infection (24). Genome sequencing and data processing were performed as previously described (12). Genome sequences have been deposited in the Short Read Archive (SRA) under BioProject accession number PRJNA255899. All putative polymorphisms identified in the *hasA* and *hasB* genes were confirmed using Sanger sequencing.

Hyaluronic acid assays. A colorimetric assay using known concentrations of a hyaluronic acid standard were used to quantify hyaluronic acid

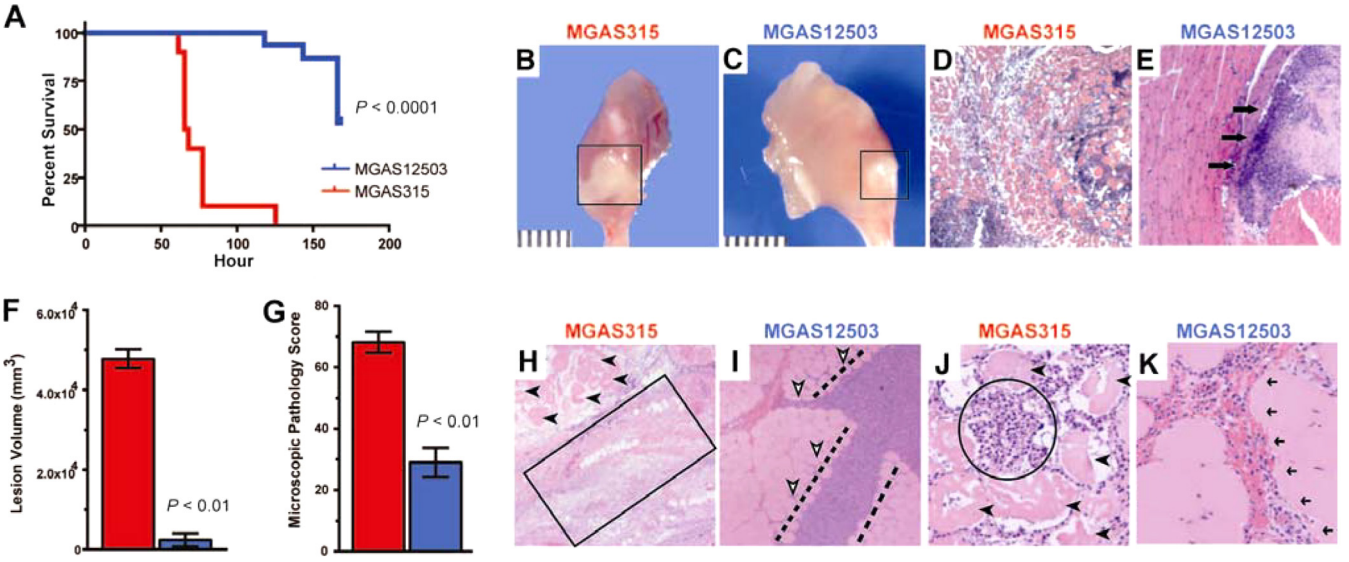

FIG 1 The MGAS12503 carriage strain is significantly less virulent than invasive GAS in necrotizing fasciitis models of GAS infection. (A) Survival curve of mice infected with carriage strain MGAS12503 and invasive strain MGAS315. Ten mice in each group were infected intramuscularly with 1×10^7 CFU, as described in Materials and Methods. *P* value is relative to MGAS315 as determined by log rank test. Gross pathology (B and C) and histopathology (D and E) (×2 original magnification) of mouse hind limb lesions at 48 h postinfection. Mice infected with strain MGAS315 had extensive spreading myonecrosis and tissue destruction (B, D). In contrast, the lesions in mice challenged with the carrier strain (C, E) had small abscesses confined to the inoculation site. The boxed area and arrows demarcate a circumscribed, walled-off lesion. (F) Cynomolgus macaques were inoculated intramuscularly in the anterior thigh ($n = 3$ monkeys per strain treatment group), and nonviable lesion volume was measured. (G) Microscopic pathology was scored using objective criteria as described in Materials and Methods. (H) Specimens taken from the inoculation site of monkeys infected with invasive strain MGAS315 show extensive necrosis (boxed region) with complete obliteration of the muscle fascicles and intervening fascial planes (original magnification, ×4). Many nonviable cells (black arrowheads) are seen in the background of necrotic debris. (I) In contrast, monkeys infected with carriage strain MGAS12503 have necrosis that is limited to the fascial planes (area between dashed lines), sparing the adjacent muscle tissue, which retains viability (white arrowheads). (J) At higher magnification (×10 original magnification), the tissue from animals infected with strain MGAS315 is completely nonviable (black arrowheads) and extensively infiltrated by PMN leukocytes (circled region). (K) In comparison, muscle tissue from animals infected with strain MGAS12503 was relatively preserved, with a very limited pattern of infiltration that was restricted to the major facial plane (black arrows).

in a 10-ml mid-exponential-phase broth culture (THY) as previously described (25).

Statistics. Log rank was used to test for statistical significance of mortality data. A two-tailed *t* test (unequal variance) was used to compare 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. Repeated-measure analysis of variance (ANOVA) was used to compare rates of nasopharyngeal colonization between strains. A *P* value of less than 0.05 was considered significant for all statistical tests.

RESULTS

Carriage strains are less virulent in mouse and nonhuman primate models of necrotizing fasciitis. We previously reported that serotype M3 carriage strain MGAS12503, which was isolated from an asymptomatic human carrier, had a significantly higher 90% near-lethal dose (LD_{90}) (that is, was less virulent) for mice than invasive serotype M3 strains after intraperitoneal (i.p.) inoculation (13). To study virulence attributes of the MGAS12503 carriage strain in more detail, we tested the hypothesis that this carriage strain is less virulent in a mouse model of necrotizing fasciitis (NF) (19) than invasive serotype M3 strain MGAS315. Consistent with the hypothesis and the results obtained from the mouse i.p. infection model, strain MGAS12503 was significantly less virulent in the NF model (Fig. 1A). The carriage strain produced significantly less tissue destruction and abscess formation compared to the extensive necrosis observed in mice inoculated with invasive strain MGAS315 (Fig. 1B and C). Similarly, MGAS12503 caused significantly less tissue pathology than invasive strain MGAS315 (Fig. 1D and E).

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 binds plasminogen of humans and nonhuman primates but not that of mice (26). Furthermore, recent research highlights deficiencies in murine models used in the investigation of human inflammatory diseases (27). Thus, we next tested the hypothesis that strain MGAS12503 is less virulent in a nonhuman primate model of NF (19). Consistent with the mouse data, the carriage strain was significantly less virulent in the nonhuman primate than invasive strain MGAS315, as assessed by lesion volume (Fig. 1F), pathology score (Fig. 1G), and magnitude and character of tissue destruction (Fig. 1H to K). Together, the mouse and nonhuman primate virulence studies clearly demonstrated that carriage strain MGAS12503 is less virulent than invasive strain MGAS315.

Carriage strain MGAS12503 contains a frameshift mutation in the *hasA* gene that eliminates capsule production and decreases virulence. In our DNA-DNA microarray comparative genome analysis, no polymorphisms relative to invasive strains were identified in MGAS12503 that would account for its significantly decreased virulence or carriage (13). Because of the unambiguous significantly decreased virulence phenotype, we reinvestigated the genome sequence of this strain. We hypothesized that additional polymorphisms may be identified with newer next-generation whole-genome sequencing technology. Using an Illumina instru-

ment and bioinformatic analyses (12), we discovered a previously unidentified frameshift mutation in the *hasA* gene, which encodes an enzyme required for hyaluronic acid capsule biosynthesis (28). The GAS capsule has been studied extensively since its initial description by Bordet in 1907 (29). Very early studies demonstrated that the GAS capsule is composed of hyaluronic acid, is nonimmunogenic, and is a major virulence factor contributing to resistance to phagocytosis by human polymorphonuclear leukocytes (PMNs) (29–31). Subsequently, the *hasA* and *hasB* genes were shown to encode enzymes required for capsule biosynthesis (32). The frameshift mutation in strain MGAS12503 results in a premature stop codon that truncates the HasA protein by 104 amino acids (Fig. 2A).

Thus, we hypothesized that this mutation results in loss of capsule biosynthesis, thereby contributing to decreased virulence and the carriage phenotype. Further, we hypothesized that repair of the mutation in *hasA* would restore capsule production and increase virulence. To test these hypotheses, we generated an isoallelic mutant strain differing from parental strain MGAS12503 only by the presence of a functional *hasA* gene. The isoallelic mutant strain (MGAS12503*hasA*^{wt}) produced significantly more hyaluronic acid than wild-type carriage strain MGAS12503, an organism that produced no detectable hyaluronic acid (Fig. 3A).

To test the altered-virulence hypothesis, we used a mouse model of NF (19). As anticipated, "repaired" strain MGAS123503*hasA*^{wt} was significantly more virulent than carriage strain MGAS12503 (Fig. 3B). Consistent with increased virulence, animals injected with strain MGAS12503*hasA*^{wt} had significantly more extensive necrosis and tissue pathology (Fig. 3C and E) than acapsular carriage strain MGAS12503 (Fig. 3D and F). As noted previously, GAS is a human-specific pathogen, and the possibility exists that the virulence differences observed in mice are not seen in a human system. Inasmuch as the hyaluronic acid capsule is known to impart resistance to phagocytosis by PMNs (29–31), we used a bactericidal assay to test the hypothesis that restoration of capsule production increased survival of the isoallelic mutant (that is, the strain with a wild-type "repaired" *hasA* allele) in human blood. We observed significantly increased (>100-fold, $P = 0.01$) survival of repaired strain MGAS12503*hasA*^{wt} compared to that of isoallelic carriage strain MGAS12503 containing the mutant *hasA* allele (Fig. 3G). The identification of the frameshift inactivating mutation in the *hasA* gene in carriage strain MGAS12503 provides a mechanism for the early observations linking decreased capsule production and carriage (33, 34).

Restoration of capsule production by carriage strain MGAS12503 increases bacterial burden in the mouse oropharynx but decreases epithelial cell internalization. The studies thus far have compared the virulence (i.e., invasive potential) of the carriage strain relative to an invasive strain and an isoallelic-derived strain. We next tested the hypothesis that the carriage strain and repaired isoallelic mutant strain (MGAS12503*hasA*^{wt}) differed significantly in ability to colonize the mouse oropharynx. Consistent with our hypothesis, isoallelic strain MGAS12503*hasA*^{wt} colonized the mouse oropharynx with a significantly greater bacterial burden ($P < 0.0001$; Fig. 4A) than the MGAS12503 parental strain cultured from the human carrier. In fact, the bacterial burden of mice colonized with the repaired MGAS12503*hasA*^{wt} strain was closely similar to that observed for the invasive MGAS315 strain (Fig. 4).

Pharyngeal epithelial cell internalization has been suggested as a mechanism for failure to eradicate GAS from the throat following antibiotic therapy (35). Further, it has been shown that GAS isolated from patients with eradication failure have greater ability to internalize epithelial cells than GAS from patients with successful eradication (36). However, the mechanism by which GAS persist in such cases is unknown. We hypothesize that the loss of capsule in MGAS12503 enhances the ability to internalize into human epithelial cells. To test this hypothesis, we performed internalization assays in cultured human epithelial cells (HaCaT) using invasive strain MGAS315, carrier strain MGAS12503, and repaired carrier strain MGAS12503*hasA*^{wt}. Consistent with our hypothesis, we observed significantly greater ability of the capsule-negative carrier MGAS12503 strain to internalize epithelial cells than either MGAS315 or the repaired isoallelic mutant (Fig. 4B).

Mutations that ablate capsule biosynthesis are present *in vivo* in humans. The mutation in *hasA* was identified in a carriage strain isolated from a healthy human with no recent history of pharyngitis (14). Our data suggest that the GAS capsule contributes to increased bacterial burden during acute infection, and subsequent loss of capsule production contributes to persistence during asymptomatic carriage. These data are consistent with the studies by Hamburger et al. (37) in that individuals with high GAS burdens are most likely to transmit to others and that the GAS bacterial burdens decreased over the course of infection and usually were the same M protein serotype (38). Thus, it is possible, and we believe likely, that the carriage strains typically represent lineal descendants of the strain causing acute pharyngitis in the same individual, rather than new acquisition via transmission from another carrier.

To directly test the hypothesis that mutations in the hyaluronic acid synthesis genes arise naturally *in vivo* in humans, we compared the genome sequences of M3 strains cultured serially from nine unrelated individuals over time (24). Whole-genome sequence data were obtained from a total of 37 GAS strains. We discovered that strains from 4 of the 9 individuals studied (44%) developed mutations over time in the *hasAB* genes, essential for capsule biosynthesis (Fig. 2B). GAS isolates cultured from a single individual were very closely related, differing on average by only 4 core genome polymorphisms, which means they are highly unlikely to represent reinfection with a new (unrelated) strain. Relative to the initial strain, strains subsequently isolated from subject A contained either one nonsynonymous nucleotide substitution in *hasB* (resulting in a P252L replacement in a highly conserved amino acid) or a nucleotide insertion in either *hasA* or *hasB* (Fig. 2A and B). Similarly, organisms from subject B contained a nucleotide insertion in *hasA*, and bacteria from subject C contained a nucleotide insertion in *hasB* (Fig. 2A and B). Each nucleotide insertion resulted in a shifted reading frame and is predicted to truncate the resulting protein and ablate capsule biosynthesis in these strains, as observed in strain MGAS12503. Two isolates from subject D have a nonsynonymous nucleotide substitution in *hasB* (D397N; also conserved among sequenced GAS strains) (Fig. 2A and B). Consistent with the identified mutations negatively affecting GAS capsule expression, we observed significantly reduced hyaluronic acid production and survival in human blood in each representative isolate from each subject (Fig. 2C and D). These findings are consistent with a recent study identifying mutations that negatively affect capsule production in GAS carrier strains isolated during a longitudinal nonhuman primate study (12). Also of note, different strains cultured longitudinally from subject A had three mutually exclusive, independent mutations in

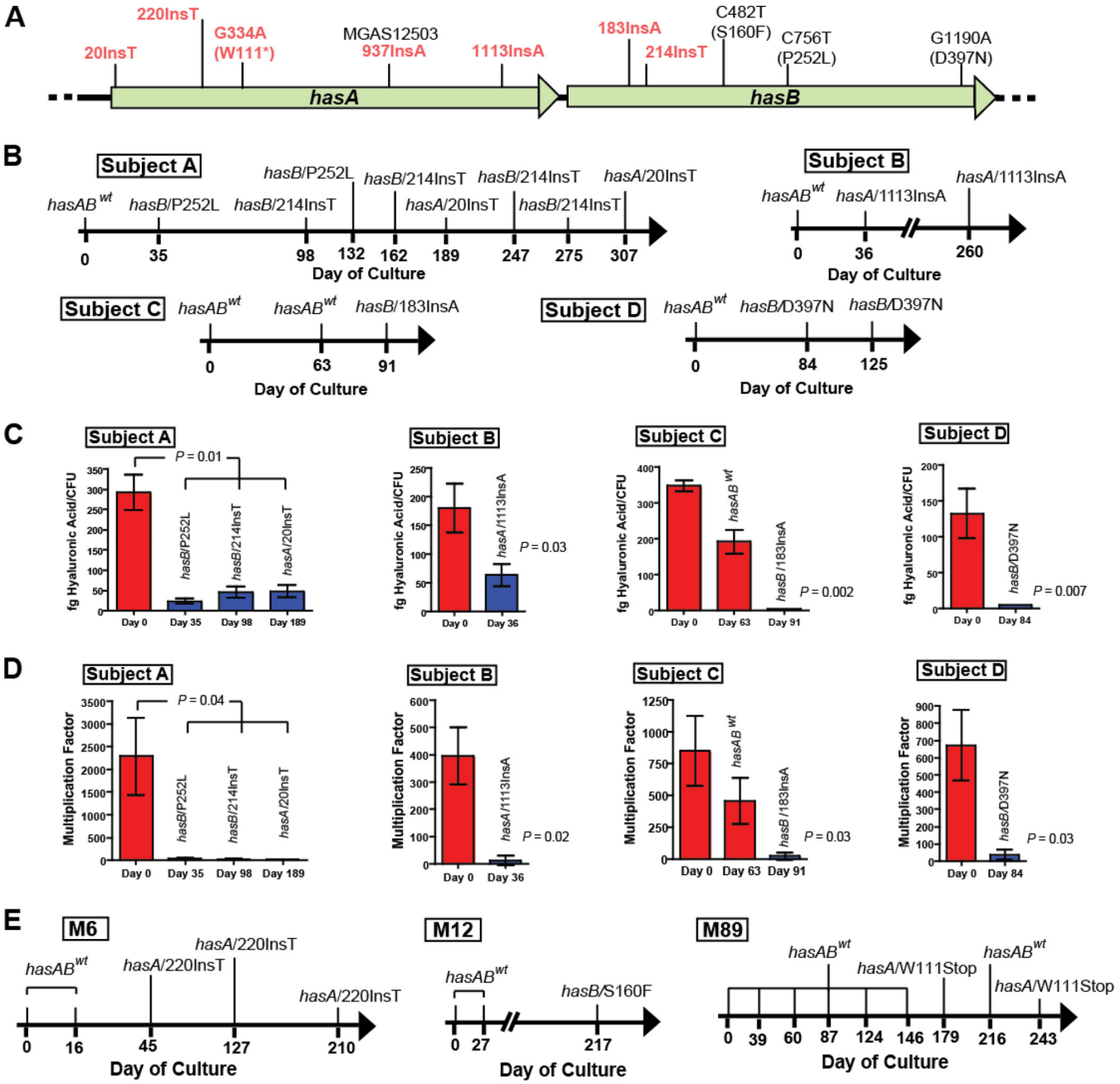

FIG 2 Location of *hasA* and *hasB* mutations identified in serially cultured GAS from humans. (A) The *hasA* and *hasB* genes are adjacent to each other in the *hasABC* operon. Strain MGAS12503 contains a nucleotide insertion at position 937 in *hasA*. Locations of nucleotide insertions (Ins) identified in other human carriage strains are shown. Mutations resulting in truncation of the predicted protein are in red text. (B) Strains of GAS serotype M3 containing mutations in either *hasA* or *hasB* are plotted according to subject and day cultured after initial isolate (day 0). In all subjects, day 0 represents first acquisition of GAS. (C) Hyaluronic acid assays of the initial isolates and representative subsequent isolates of serotype M3. Assays were performed in triplicate as described in Materials and Methods. Error bars represent standard deviations. *P* values were determined by *t* test (unequal variance). fg, femtograms. (D) Multiplication of initial isolate and representative subsequent isolates of serotype M3 GAS in human blood. Human blood was inoculated with the indicated strains as described in Materials and Methods. Shown is multiplication after growth in blood from a single donor in triplicate. *P* value determined by *t* test (unequal variance). (E) Strains of GAS serotype M6, M12, and M89 containing mutations in either *hasA* or *hasB* are plotted according to subject and day cultured after initial isolate, as described for panel B.

the hyaluronic acid capsule biosynthesis genes (Fig. 2A and B), further emphasizing the link between capsule downregulation and GAS carriage.

Mutations in capsule biosynthesis genes arise *in vivo* in multiple GAS M protein serotype human isolates. It is possible that the mutations in the capsule biosynthesis genes observed thus far are unique to serotype M3 GAS. Thus, we next sought to test the hypothesis that capsule-ablating mutations commonly arise in other GAS serotypes during carriage. Whole-genome sequence data were obtained from an additional 44 GAS strains represent-

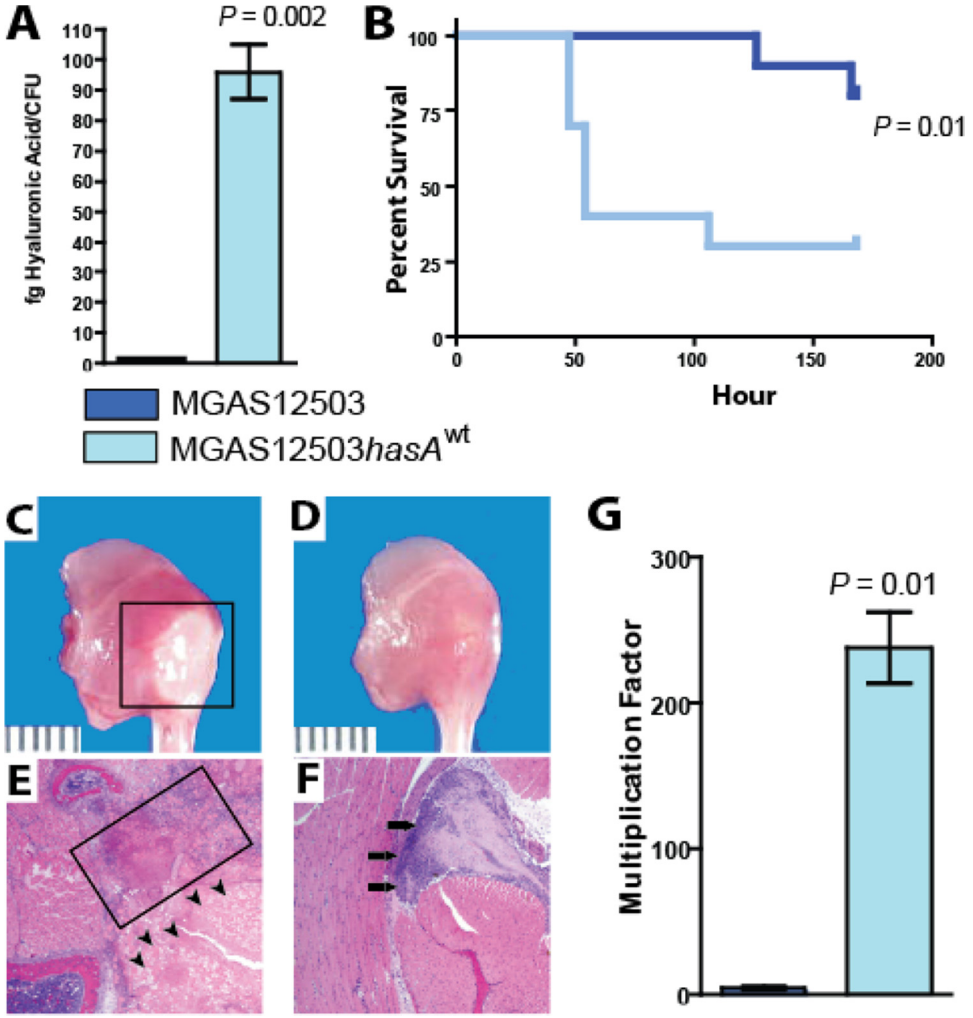

FIG 3 Repair of the *hasA* mutation in strain MGAS12503 restores capsule production and virulence. (A) Hyaluronic acid assays of MGAS12503 and repaired strain MGAS12503*hasA*^{wt}. Error bars represent standard deviations after performing assays on three biologic replicate samples. Strain MGAS12503 did not produce detectable hyaluronic acid. fg, femtograms. (B) Survival curve of mice infected with MGAS12503 or repaired strain MGAS12503*hasA*^{wt}. Ten mice in each group were infected intramuscularly with 1×10^7 CFU, as described in Materials and Methods. *P* values determined by log rank test. (C and D) Mice infected with MGAS12503*hasA*^{wt} had significantly greater myonecrosis at the site of infection than mice infected with MGAS12503 containing the mutant *hasA* allele. (E and F) Extensive tissue destruction (boxed area) and nonviable cells were observed in the mouse infected with strain MGAS12503*hasA*^{wt}, whereas animals infected with MGAS12503 had only small abscesses (black arrows). (G) Multiplication of MGAS12503 and MGAS12503*hasA*^{wt} in human blood. Whole blood was inoculated with the indicated strains as described in Materials and Methods. Shown is multiplication after growth in blood from a single donor. Similar results were obtained with additional donors. Error bars indicate standard deviations of growth performed in triplicate. *P* values were determined by *t* test (unequal variance).

ing 10 epidemiologic-independent individuals (in some instances cultured over 8 months) and 3 GAS M types (Fig. 2E). In total, the GAS serotypes examined (M3, M6, M12, and M89) represent approximately 35% of all serotypes reported to cause pharyngitis in the United States and Canada (39, 40). Similar to serotype M3 GAS, isolates cultured from a single individual were very closely related, with an average core genome single-nucleotide polymorphism (SNP) difference ranging from a low of 1.6 (M6) to a high of 53.6 (M89). We discovered that, relative to the initially cultured strain, subsequent isolates from 3 of the 10 subjects developed mutations in *hasA* or *hasB* that are predicted to negatively affect the GAS hyaluronic acid capsule (Fig. 2E). Consistent with the identified carriage-associated mutations negatively affecting GAS capsule expression, we identified significantly reduced hyaluronic acid production in representative isolates harboring mutations in the *hasAB* genes relative to the initially infecting strain from the corresponding subject (data not shown). In summary, GAS strains from 7 of 19 (37%) epidemiologically independent subjects studied developed mutations during the course of carriage that reduced or eliminated capsule biosynthesis. Thus, it is clear that mutations reducing capsule biosynthesis are the dominant genetic change in this cohort of multiple GAS serotypes that are common causes of pharyngitis and other infections. In combination with the virulence and colonization data, our data definitively demonstrate that mutations negatively affecting capsule biosynthesis arise over time in the posterior pharynx of humans.

DISCUSSION

The past several decades have seen dramatic advances in understanding how bacterial pathogens cause a diverse array of disease

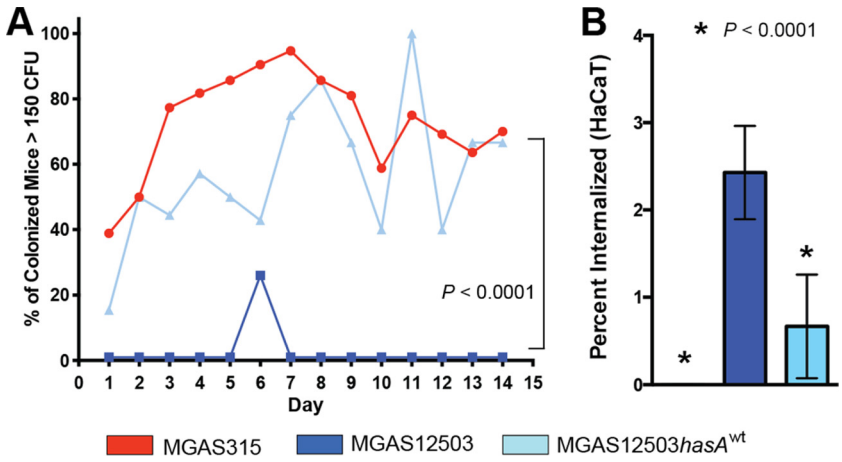

FIG 4 Capsule contributes to bacterial burden in mice but decreases ability to internalize epithelial cells. (A) Nasopharyngeal colonization of mice with GAS strains. Percent of colonized mice with >150 CFU isolated after daily swabbing for each strain: MGAS315 (invasive), MGAS12503 (carrier), and MGAS12503*hasA*^{wt} (repaired carrier). Mice were inoculated intranasally and swabbed daily for 14 days as described in Materials and Methods. *P* values were determined by repeated-measures ANOVA. (B) Internalization of MGAS315, MGAS12503, and MGAS12503*hasA*^{wt} into cultured human epithelial cells (HaCaT) as described in Materials and Methods. Less than 0.1% of strain MGAS315 internalized epithelial cells. *P* values are relative to the carrier strain MGAS12503 and determined by *t* test (unequal variance).

in humans. In contrast, little attention has been given to the study of molecular factors contributing to carriage, despite the fact that for many bacterial pathogens it is a far more common lifestyle than clinically significant infection. We have discovered that mutations resulting in loss of capsule biosynthesis arise during adaptation of GAS to the human pharynx. Our findings echo recent reports that *Pseudomonas aeruginosa* (41) and *Burkholderia dolosa* (42) strains in chronically colonized cystic fibrosis patients have mutations in genes encoding known and potentially novel virulence factors or virulence factor regulators.

Our findings are consistent with early studies that reported GAS throat isolates cultured from asymptomatic humans lack observable capsule (33, 34) and were subsequently shown to produce less hyaluronic acid capsule than infecting strains of GAS (7). The identification of frameshift-inactivating mutations in the *hasA* or *hasB* genes in carrier strains provides a molecular mechanism for the early observations linking decreased capsule production and carriage. Unencapsulated strains of other bacterial pathogens, such as *N. meningitidis* (43), *S. pneumoniae* (44), *Streptococcus agalactiae* (group B streptococcus) (45), and *Haemophilus influ-*

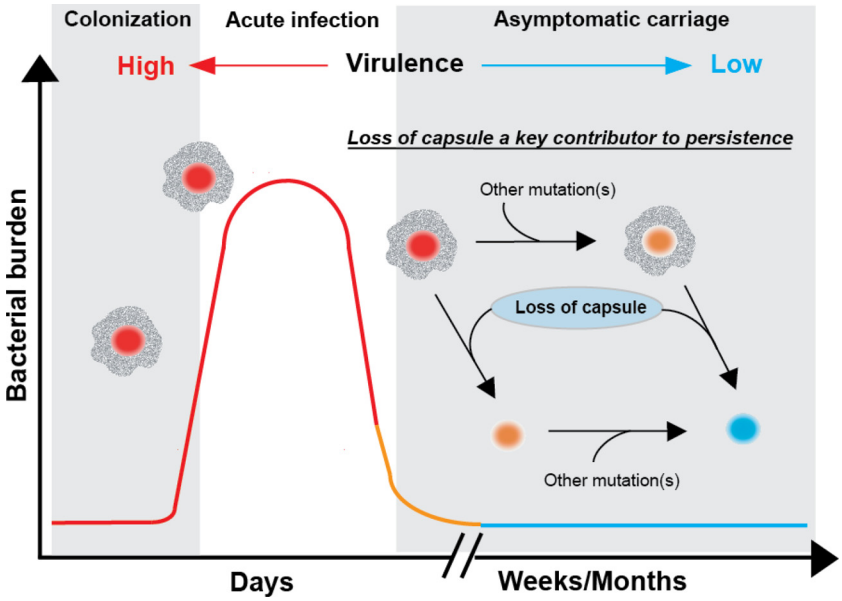

FIG 5 Loss of hyaluronic acid capsule is a key contributor to GAS asymptomatic carriage. Three stylized phases of GAS pharyngeal infection are shown, based on Virtaneva et al. (58). Bacterial numbers are highest during the acute infection phase (37, 58). In acute infection, encapsulated GAS strains with higher virulence (red circles) predominate. During asymptomatic carriage, selection of GAS strains with mutations negatively affecting capsule takes place along with additional undefined mutations. Loss of capsule can occur early or after other mutations have already occurred, ultimately resulting in loss of virulence (blue circles) and adaptation to the human host.

enzae (46), have been frequently isolated from carriers. More recently, it has been shown that *S. pneumoniae* differentially regulates capsule production in a niche-specific manner such that decreased capsule production promotes colonization (47). Thus, decreasing or eliminating capsule production in multiple bacterial pathogens appears to offer an advantage to persistence on mucosal surfaces.

The data begin to unravel the molecular genetic basis underlying the decades-old observation that asymptomatically carried GAS strains are often unencapsulated. It is possible that immune evasion by masking of surface proteins through increased capsule production (48) and active participation of capsule in adherence to epithelial cells (49, 50) contribute to the observed differences in colonization of the oropharynx between the encapsulated and unencapsulated strains. However, other studies have shown that compared to poorly encapsulated GAS, encapsulated strains have reduced adherence to and internalization by epithelial cells (25). This phenomenon has also been observed in *H. influenzae* (51) and *S. pneumoniae* (52) strains lacking capsule. Some investigators have postulated that adherence and subsequent internalization of GAS into epithelial cells contributes to persistent colonization (53) and failure to eradicate GAS after treatment of acute pharyngitis (35). Very recent studies provide evidence that GAS may not only survive epithelial internalization (54) but, under some circumstances, escape autophagy and replicate within host epithelial cells (55). Survival within epithelial cells may provide GAS carrier strains with a "safe haven" by limiting antibiotic and immune system exposure. It could be that increased capsule production may contribute to acute symptomatic infection, but as symptoms abate, loss of capsule allows for greater adherence and internalization, signifying adaptation to the human host and persistence.

These findings should not be interpreted to mean that mutations that reduce or ablate capsule synthesis are the sole molecular events responsible for carriage in all strains. Clearly it is possible, and expected, that mutations in other genes that detrimentally alter virulence could in principle contribute to the carrier phenotype in GAS (Fig. 5) and other bacterial pathogens. For instance, early studies reported a decrease in M protein in GAS strains isolated from carrier strains compared to that in organisms recovered from acute infection (56, 57). Consistent with this, we previously reported that a serotype M3 GAS carrier isolate had a 195-bp deletion in *emm*, the gene encoding M protein, that removed the hypervariable N terminus (13). The strains examined in this study did not contain polymorphisms in *emm* predicted to alter M protein production. Thus, the data indicate that mutations eliminating capsule production are the single predominant category of polymorphisms in organisms serially cultured from the pharynx. Our findings suggest a common mechanism contributing to the numerically frequent carriage phenotype of organisms generally considered to be frank pathogens.

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