Growth Characteristics of and Virulence Factor Production by Group A *Streptococcus* during Cultivation in Human Saliva

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Group A *Streptococcus* (GAS) commonly infects the human oropharynx, but the initial molecular events governing this process are poorly understood. Saliva is a major component of the innate and acquired immune defense in this anatomic site. Although landmark studies were done more than 60 years ago, investigation of GAS-saliva interaction has not been addressed extensively in recent years. Serotype M1 GAS strain MGAS5005 cultured in human saliva grew to $\sim 10^7$ CFU/ml and, remarkably, maintained this density for up to 28 days. Strains of several other M-protein serotypes had similar initial growth patterns but did not maintain as high a CFU count during prolonged culture. As revealed by analysis of the growth of isogenic mutant strains, the ability of GAS to maintain high numbers of CFU/ml during the prolonged stationary phase in saliva was dependent on production of streptococcal inhibitor of complement (Sic) and streptococcal pyrogenic exotoxin B (SpeB). During cultivation in human saliva, GAS had growth-phase-dependent production of multiple proven and putative extracellular virulence factors, including Sic, SpeB, streptococcal pyrogenic exotoxin A, Mac protein, and streptococcal phospholipase A₂. Our results clearly show that GAS responds in a complex fashion to growth in human saliva, suggesting that the molecular processes that enhance colonization and survival in the upper respiratory tract of humans are well under way before the organism reaches the epithelial cell surface.

One hallmark of a successful microorganism is the ability to adapt to new host environments. Group A *Streptococcus* (GAS) causes a wide variety of diseases in humans, ranging from impetiginous skin lesions to invasive diseases, such as necrotizing fasciitis and meningitis. GAS is particularly suited to inhabit the human oropharynx, colonizing as many as one-half of school-age children in nonepidemic periods and causing an estimated 15 million cases of pharyngitis in the United States each year (3, 43). Moreover, the presence of GAS in the oropharynx generally is required for the subsequent development of rheumatic fever, the leading cause of preventable heart disease in children (10, 28).

The oropharynx is the major site of entry for GAS into the human body and its main portal of transmission (19, 43, 46). Saliva is ubiquitous in the human oropharynx and is an essential part of both the acquired and innate immune defense systems (25, 39). Landmark experiments conducted more than 60 years ago demonstrated the important role played by saliva in the establishment of GAS infection and the subsequent transmission of infectious organisms (18–20). These studies revealed that large numbers of live GAS were present in the saliva of individuals with GAS pharyngitis (19). Moreover, it was established that a major route for dissemination of GAS from infected individuals into the environment was via dispersal of aerosolized saliva (20). Other investigators have reported that pharyngitis patients with detectable levels of GAS

in their saliva were more likely to transmit the organism than were individuals whose saliva did not contain GAS (26, 58). These observations, together with the known importance of saliva in host defense, identify the crucial significance of the GAS-saliva interaction. Despite this knowledge, the GAS-saliva interaction has not been extensively investigated in recent years.

To increase our understanding of this interaction, we studied the growth of GAS in saliva collected from healthy human volunteers. We used genetically diverse GAS strains to determine whether the ability of GAS to grow in saliva is widespread within the species. Western immunoblot analysis was used to test the hypothesis that proven and putative extracellular virulence proteins are produced during cultivation of GAS in saliva and to examine the relationship between virulence factor production and growth phenotypes. Finally, isogenic mutant strains were analyzed to determine whether inactivation of genes encoding extracellular virulence factors or transcriptional regulators altered the growth pattern of GAS in saliva.

MATERIALS AND METHODS

Bacterial strains and culture media. Serotype M1 strain MGAS5005 has been extensively characterized and used for in vitro and animal models of GAS infection (24, 35, 50, 51, 55, 57). The other bacterial strains used in this study are listed in Table 1. GAS was grown on Trypticase soy agar containing 5% sheep blood agar (BSA) (Becton Dickinson, Cockeysville, Md.) in Todd-Hewitt broth containing 0.2% (wt/vol) yeast extract (THY) (Difco Laboratories, Detroit, Mich.) or in a commercially available chemically defined medium (CDM) (JR Biosciences, Denver, Pa.) (56).

Isogenic mutant strains of GAS. The isogenic mutant strains used in this study were created previously by nonpolar insertional mutagenesis as described previously (12, 35, 37). The parental strain used was serotype M1 strain MGAS5005, except for the isogenic *irr* mutant strain JRS550, which was derived from serotype

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TABLE 1. Bacterial strains used in this study

Strain ^a	M serotype	Yr isolated	Disease or source
MGAS5005	1	1996	Cerebrospinal fluid
SF370	1	1985	Wound infection
MGAS5475	1	1994	Pharyngeal
MGAS9415	1	2001	Pharyngeal
MGAS11705	1	2003	Pharyngeal
MGAS315	3	1988	Toxic shock
JRS4 ^b	6	1971	Unknown
MGAS9645	6	2001	Pharyngeal
MGAS10287	6	2002	Pharyngeal
MGAS12487	9	2003	Pharyngeal
MGAS12292	11	2003	Pharyngeal
MGAS9429	12	2001	Pharyngeal
MGAS6180	28	1998	Blood
MGAS9530	33	2001	Pharyngeal
MGAS9464	43	2001	Pharyngeal
MGAS12083	73	2003	Pharyngeal
MGAS9482	77	2001	Pharyngeal
MGAS12349	94	2003	Pharyngeal

^a MGAS, Musser group A *Streptococcus*.

^b Obtained from J. R. Scott.

M6 strain JRS4, and the isogenic *sla* mutant strain, which was derived from serotype M3 strain MGAS315 (34, 35). The isogenic mutant strains were analyzed by Southern hybridization and DNA sequencing to confirm that the proper genetic constructs were made (37, 38). The absence of specific gene expression and protein production by the mutant strains was confirmed by Northern blot and Western immunoblot analysis, respectively (32, 36).

Collection of saliva from healthy human volunteers. Saliva was collected on ice from healthy volunteers under a Baylor College of Medicine Institutional Review Board human subjects protocol using a modification of the method

described by DeJong et al. (8). Saliva production was stimulated by chewing on paraffin wax, and 2.5 mmol of dithiothreitol (DTT) (Fisher Scientific, Pittsburgh, Pa.) was added to aid subsequent filtration. The saliva was clarified by centrifugation at 40,000 × *g* for 15 min and sterilized by filtration through a 0.20-μm filter (Nalgene Labware, Rochester, NY). The saliva was frozen at −20°C and thawed immediately prior to use. Pooled saliva collected from at least four donors was used to minimize effects of donor variation on study results.

Growth of GAS in human saliva. Bacteria were incubated at 37°C with 5% CO₂. A schematic of the procedures used to grow GAS is shown in Fig. 1. GAS was grown overnight on BSA to confirm colony morphology and purity. Bacteria were inoculated into THY, grown overnight, diluted 1:50 with fresh THY broth, and grown to mid-logarithmic phase (optical density at 600 nm [OD₆₀₀] of ~0.5). A 1:50 dilution of the mid-logarithmic-phase culture was made into 10 ml of saliva to create saliva medium A. After 4.5 h of growth in saliva medium A, a second 1:50 dilution was made in 10 ml of fresh saliva (saliva medium B). Aliquots were removed from both saliva media every 1.5 h for the first 9 h and then daily thereafter and assessed for both OD₆₀₀ and number of CFU. For determination of CFU, samples were serially diluted 10-fold in phosphate-buffered saline (PBS) (Sigma) and plated in duplicate on BSA. The plates were incubated overnight, and colonies were counted. Each experiment was performed at least in quadruplicate.

Western immunoblot analysis. GAS were grown in saliva medium, and the supernatants obtained at each time point were adjusted to be derived from 5 × 10⁸ CFU. The culture supernatant was collected by centrifugation at 3,000 × *g* for 10 min. Saliva without GAS added was used as the negative control. The supernatant proteins were precipitated with 30% trichloroacetic acid (Sigma) on ice for 30 min, and the precipitates were collected by centrifugation at 17,000 × *g* for 15 min. The pellets were washed twice in ice-cold acetone (Sigma), dried in a speed vacuum for 10 min, and suspended in 0.1 ml of EB buffer (QIAGEN). The proteins present in the concentrated supernatants were separated with sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (Bio-Rad) and transferred (TransBlot; Bio-Rad) to nitrocellulose membranes in Towbin's buffer at 15 V for 1 h. Purified recombinant proteins were included in the Western immunoblot analyses as positive controls (24, 31). The membranes were incubated overnight in 1× Tris-buffered saline (TBS) containing 5% Liquid

FIG. 1. Schematic of the experimental strategy used to analyze growth of GAS in human saliva. GAS strains were grown to mid-exponential phase (OD₆₀₀, ~0.5) in THY broth and diluted 1:50 in saliva medium A. After 4.5 h of growth in saliva medium A, a second 1:50 dilution was performed in saliva medium B. Samples were removed every 1.5 h for 9 h and then every 24 h for OD₆₀₀ readings and CFU analysis.

Block (TBSB) (Amersham Pharmacia, Buckinghamshire, United Kingdom) and incubated for 1 h in TBSB plus primary antibody (1:10,000) (2, 32). The membranes were washed three times in PBS plus 0.1% Tween 20 (Bio-Rad), incubated for 1 h with secondary antibody (1:2,500; goat antirabbit-conjugated horseradish peroxidase; Pierce), and washed three times in PBS plus 0.1% Tween 20. The membranes were incubated with Super Signal West Pico chemiluminescent substrate reagent (Pierce) according to directions suggested by the manufacturer, exposed to HyperFilm (Amersham Pharmacia), and developed.

Addition of purified proteins to saliva. Streptococcal inhibitor of complement (Sic) and streptococcal pyrogenic exotoxin B (SpeB) were cloned, overexpressed, and purified as described previously (23, 27). The purified proteins were added as appropriate to saliva at physiologic concentrations, 5 μ g/ml for Sic and 20 μ g/ml for SpeB (16, 52).

Statistical analysis. Differences between GAS strains grown in saliva were analyzed by determining the concentration ($\log_{10}$ CFU/ml) at baseline and at maximum growth in saliva medium B for each strain and then using a paired Student's *t* test to compare values. The ability of a particular GAS strain to persist in saliva was analyzed using a paired Student's *t* test to compare concentrations ($\log_{10}$ CFU/ml) in saliva medium B at 168 h. Statistical significance was assigned at a two-sided *P* value of 0.05 using Bonferroni's adjustment for multiple comparisons.

RESULTS

Inability to correlate OD_{600} values with CFU during growth in saliva. Because obtaining CFU is time consuming, labor intensive, and expensive, most investigators use OD_{600} readings as a surrogate marker to analyze bacterial growth. However, in preliminary experiments, we were not able to reproducibly correlate OD_{600} readings with CFU concentrations obtained by serial plating. Most OD_{600} values were quite low, and we observed decreased OD_{600} values despite increased CFU and vice versa. This finding has been reported previously for other organisms and is likely due to the formation of bacterial aggregates in the saliva medium (8, 33, 42). Therefore, all bacterial growth data are expressed as numbers of CFU.

Growth of serotype M1 strain MGAS5005 in saliva. We used serotype M1 strain MGAS5005 for our initial growth studies because it is genetically representative of M1 strains that cause a high proportion of invasive and noninvasive GAS infections and its genome has recently been sequenced (24, 40, 51). This strain's growth pattern in saliva was highly reproducible (Fig. 2A). There was a rapid increase of 1 to 1.5 $\log_{10}$ CFU/ml when mid-exponential-growth-phase organisms were transferred to saliva. A peak value of 2.5×10^7 CFU/ml was reached within a few hours. Surprisingly, a prolonged stationary phase was observed, characterized by a cell density of $\sim 1 \times 10^7$ CFU/ml that persisted for up to 28 days (Fig. 2B).

We hypothesized that the initial rapid growth in saliva was due to consumption of residual THY broth carried over during subculture of the bacteria. To test this hypothesis, we subjected organisms that had been grown in saliva medium A for 4.5 h to a second 1:50 dilution in fresh saliva (saliva medium B). The density of organisms grown in saliva medium B increased by ~ 1.5 to $2.0 \log_{10}$ CFU/ml over the next 24 h, despite starting from an inoculum that was nearly $1.0 \log_{10}$ less than the starting inoculum in saliva medium A. The resulting density ($\sim 1 \times 10^7$ CFU/ml) was essentially identical to that of GAS grown in saliva medium A. In addition, a similar prolonged stationary phase was observed in saliva medium B. We next determined the ability of serotype M1 strain MGAS5005 to grow in response to a fresh nutrient supply added after prolonged culture. Organisms growing in saliva for 7 days were subcultured

FIG. 2. Growth patterns of serotype M1 strains in various media. Growth in saliva was assayed by subculturing exponential-growth-phase organisms from THY 1:50 into saliva (saliva medium A). After 4.5 h in saliva medium A, organisms were subcultured 1:50 into saliva medium B. Aliquots were removed at indicated time points, and numbers of CFU were determined after overnight incubation on BSA at 37°C in 5% CO₂. (A) Growth over 7 days for serotype M1 strain MGAS5005. Symbols: Saliva medium A, ▲, dashed line; saliva medium B, ■, solid line; THY medium, □, dashed line; CDM, ●, solid line; saliva plus glucose, ◆, dashed line; saliva with pH controlled, X, solid line. (B) Growth over 28 days (d) for five serotype M1 strains in saliva medium B. Symbols: MGAS5005, ■, solid line; SF370, ▲, dashed line; MGAS5474, ▼, solid line; MGAS9415, ◆, dashed line; MGAS11705, ●, solid line. Bars represent standard deviations.

at a 1:50 dilution into fresh saliva. The organisms grew $\sim 2.0 \log_{10}$ CFU/ml to reach $\sim 10^7$ CFU/ml within 18 h. Together, these data indicate that serotype M1 strain MGAS5005 not only grew in human saliva to a cell density of $\sim 10^7$ CFU/ml but also maintained the ability to proliferate in response to a fresh supply of saliva, even after a prolonged stationary phase.

Addition of glucose or control of pH did not alter the saliva growth phenotype. Because the concentration of glucose in human saliva is only about 1% of that in blood or tissue, it has been suggested that the level of free glucose in saliva is insufficient to support bacterial cell division to values exceeding 1×10^7 CFU/ml (42, 48). We added exogenous glucose to saliva to achieve glucose levels equal to that found in human blood (100 mg/dl) but observed no difference in the maximum density of GAS (Fig. 2A). The normal pH of human saliva is between 6 and 7, and the presence of bicarbonate provides significant buffering capacity (25). The pH of the saliva during growth of

GAS ranged between 6.5 and 7.5. We used exogenous bicarbonate to maintain the culture media between pH 7 and 7.1 but observed no difference in maximum growth achieved by GAS in the pH-stabilized saliva compared with saliva where no effort was made to control pH (Fig. 2A). Finally, we were able to filter a limited amount of saliva without adding DTT. Serotype M1 strain MGAS5005 grown in saliva filtered without DTT had no change in growth phenotype compared to results when the usual DTT-filtered saliva was used. Taken together, we conclude that glucose levels, pH, or the addition of DTT did not affect the growth phenotype of serotype M1 strain MGAS5005 in human saliva.

Prolonged stationary phase not observed in other laboratory media. The steady, high-density persistence of serotype M1 strain MGAS5005 in saliva for at least 28 days was very unexpected. We studied the growth of serotype M1 strain MGAS5005 in nutrient-rich laboratory media (THY) and in nutrient-limited CDM. In both THY and CDM, there was a rapid decline of viable organisms after the first 24 h, and we were unable to detect viable organisms after more than 5 days (Fig. 2B). These results indicate that the interaction of serotype M1 strain MGAS5005 with saliva led to the unusually prolonged stationary phase.

Growth of GAS in saliva leads to decreased killing by ampicillin. To achieve a prolonged steady cell density during cultivation in saliva, serotype M1 strain MGAS5005 either had to closely balance cell death with cell renewal or had to enter a state of decreased cellular turnover. Given the limited nutrients available in saliva, we hypothesized that decreased cellular turnover was responsible for the prolonged stationary phase observed during growth in saliva. To test this hypothesis, we grew serotype M1 strain MGAS5005 in saliva for 72 h and then added various concentrations of ampicillin, a drug which kills actively dividing cells. After 72 h of growth in saliva, the minimum bactericidal concentration of ampicillin was nearly 100-fold higher than that in either THY or CDM (Fig. 3). These data support our hypothesis that cultivation of GAS in saliva leads to diminished cellular turnover.

Growth of genetically diverse GAS strains in saliva. To test the general applicability of our findings with serotype M1 strain MGAS5005, we studied four other serotype M1 strains with diverse origins (Table 1). We found that all five strains had similar growth phenotypes over 28 days (Fig. 2A). To study the applicability of our findings to GAS in general, we studied the growth of strains (Table 1) representing serotypes that commonly cause pharyngitis (M3, M6, M12, M28, and M77) and strains of serotypes that rarely cause pharyngitis (M9, M11, M33, M43, M73, and M94) (11, 21, 22, 49, 54). We hypothesized that the strains of serotypes that commonly caused pharyngitis would achieve higher maximum growth densities or persist for longer periods in human saliva than strains of serotypes that rarely cause pharyngitis.

All of the GAS strains tested grew in saliva medium B to a density of $\sim 10^7$ CFU/ml within 24 h (Fig. 4A and B). However, the ability of various strains to persist in saliva varied markedly. The serotype M1 strains persisted at the highest levels, whereas the serotype M3, M9, M11, M12, M28, M33, M73, and M77 strains persisted at slightly lower densities, $\sim 5.5 \times 10^5$ CFU/ml (Fig. 4A and B). In addition, within about 10 days we detected no viable organisms for the serotype M6 strain JRS4

FIG. 3. Time-kill analyses of serotype M1 strain MGAS5005 in various media. Bacteria were grown in saliva, THY, or CDM, and concentrations were adjusted to achieve a starting inoculum of $\sim 10^7$ CFU/ml. Ampicillin was then added at indicated concentrations, and viable organisms were assessed by serial dilutions and plating onto BSA. Symbols: Saliva without ampicillin, $\blacksquare$, solid line; saliva with 0.016 $\mu\text{g/ml}$ ampicillin, $\blacktriangledown$, dashed line; saliva with 0.512 $\mu\text{g/ml}$ ampicillin, $\triangle$, solid line; saliva with 1.024 $\mu\text{g/ml}$ ampicillin, $\bullet$, dashed line; THY with 0.016 $\mu\text{g/ml}$ ampicillin, $\square$, solid line; CDM with 0.016 $\mu\text{g/ml}$ ampicillin, $\blacklozenge$, solid line. Bars represent standard deviations.

and the serotype M43 and M94 strains (Fig. 4B). The lack of persistence of the serotype M6 strain JRS4 was surprising in that M6 serotypes commonly cause pharyngitis whereas M43 and M94 serotype strains rarely cause pharyngitis. Strain JRS4 was isolated in 1971, and hence, we hypothesized that its prolonged time in laboratory conditions had rendered it less able to grow in saliva. We tested this hypothesis by growing two other serotype M6 strains that were recently isolated (Table 1) and found that these two strains had a prolonged stationary phase compared to JRS4 (Fig. 4B). Taken together, these data show GAS strains of diverse serotypes generally achieve similar maximum densities during cultivation in saliva. There were some differences among the abilities of various GAS strains to persist in saliva, but we could draw no definitive correlation between the growth phenotype of a particular strain in saliva and the propensity of that strain's particular serotype to cause pharyngitis.

Sic is necessary for optimum growth of serotype M1 strain MGAS5005 in human saliva. Sic is an extracellular protein produced mainly by M1-serotype strains that binds to and inactivates antimicrobial peptides that are present in human saliva, such as lysozyme and β -defensins (13–16). We hypothesized that production of Sic by M1 strains was necessary for optimum growth in human saliva. Sic was produced by serotype M1 strain MGAS5005 during all phases of growth in saliva (Fig. 5). We compared the growth in saliva of an *sic* isogenic mutant strain with its parental wild-type strain. The *sic* isogenic mutant strain had decreased growth within 2 h, and its density remained lower over the entire observation period (Fig. 6; Table 2). Addition of recombinant Sic to saliva at physiologic concentrations (5 $\mu\text{g/ml}$) (16) restored the growth density to wild-type levels (Fig. 6). Taken together, we conclude that Sic is required for the optimum growth in saliva of serotype M1 MGAS5005.

FIG. 4. Growth curves of GAS strains in saliva. Growth was assayed by removing aliquots from saliva medium B at indicated time points and counting CFU after overnight incubation on BSA at 37°C in 5% CO₂. (A) Growth curves for serotype M1 strain MGAS5005 (■, solid line), serotype M3 strain MGAS315 (◆, dashed line), serotype M6 strain MGAS9645 (○, solid line); serotype M12 strain MGAS9429 (□, dashed line), serotype M28 strain MGAS6180 (●, dashed line), serotype M33 strain MGAS9530 (▲, dashed line), and serotype M73 strain MGAS12083 (△, solid line). (B) Growth curves for serotype M1 strain MGAS5005 (■, solid line), serotype M6 strain JRS4 (◆, dashed line), serotype M6 strain MGAS9645 (○, solid line), serotype M6 strain MGAS10287 (◇, solid line), serotype M9 strain MGAS12487 (X, dashed line), serotype M43 strain MGAS9464 (▼, dashed line), and serotype M94 strain MGAS12349 (□, dashed line). Results for serotype M11 strain MGAS12292 and serotype M77 strain MGAS9482 overlapped with those for serotype M6 strains MGAS9645 and MGAS10282 and the serotype M9 strain MGAS12487 and are not graphed. Bars represent standard deviations.

SpeB is necessary for optimum growth of serotype M1 strain MGAS5005 and serotype M6 strain JRS4 in human saliva.

SpeB is another GAS extracellular protein that inactivates antimicrobial peptides present in saliva (47). Unlike Sic, SpeB is produced by a wide variety of GAS serotypes, although extracellular SpeB activity levels have been reported to be highly variable (27, 52). We hypothesized that SpeB production would be needed for optimal growth in saliva. Serotype M1 strain MGAS5005 produced SpeB in a time-dependent fashion during growth in saliva (Fig. 5). We examined SpeB production during growth in saliva for each of the GAS strains studied (Table 3). Strains that were able to persist in saliva beyond 7 days produced detectable amounts of SpeB, whereas strains that did not persist in saliva did not produce detectable amounts of SpeB.

A *speB* isogenic mutant strain was used to directly test the contribution of SpeB to persistence in saliva. Similar to the *sic*

isogenic mutant strain, we observed decreased persistence of the *speB* isogenic mutant strain compared to the wild type (Fig. 6). Persistence of the mutant was restored to wild-type levels by the addition of purified SpeB to saliva. We next added purified SpeB to saliva in which the non-SpeB-producing serotype M6 strain JRS4 was growing. Following the addition of exogenous SpeB, JRS4 had a prolonged stationary phase similar to that of the SpeB-producing serotype M6 strain MGAS10287 (data not shown). Taken together, these data indicated that SpeB production is needed for optimum persistence of GAS during growth in saliva.

GAS produces a variety of extracellular virulence factors during growth in saliva. Having found that Sic and SpeB were produced during growth in saliva, we hypothesized that other proven and putative extracellular virulence proteins were produced by GAS during cultivation in saliva. Using Western immunoblot analysis, we found growth-phase-dependent production of several known and putative GAS virulence factors, such as streptococcal Mac protein (Mac), streptococcal pyrogenic exotoxin A (SpeA), streptococcal phospholipase A₂ (SlaA), and SPY2191. In light of our earlier findings with the *sic* and *speB* isogenic mutant strains, we also examined the growth of *mac*, *SlaA*, and *SPY2191* isogenic mutant strains. We found that the *mac*, *SlaA*, and *SPY2191* isogenic mutant strains and their parental wild-type strains had similar growth phenotypes (Table 2). Taken together with the Sic and SpeB protein production data, we conclude that during growth in saliva, GAS elaborates a wide range of extracellular proteins, at least two of which directly impact the ability of the organism to grow and persist in saliva.

Evaluation of effect of transcriptional regulators on growth of GAS in saliva. The entry of GAS into a prolonged stationary phase following growth in saliva combined with our finding of growth-phase-dependent production of multiple virulence factors suggests that GAS sensed and responded to the environmental conditions it encountered in saliva. To better understand how GAS altered its gene regulation during interaction with saliva, we studied the growth pattern of 11 isogenic mutant strains with deletions in various known and putative transcriptional regulators (17, 44, 57; unpublished data). In previous work, no apparent differences were identified in the growth of these isogenic mutant strains in THY compared to that of the wild type (17, 44, 57). All of the 11 isogenic mutant strains studied here had growth phenotypes similar to that of their parental wild-type strain in terms of maximum growth achieved and the ability to persist during the stationary phase (Table 2). We conclude that the 11 transcriptional regulators we studied are not crucial for maximal growth or persistence of serotype M1 strain MGAS5005 in human saliva.

DISCUSSION

Growth of GAS in human saliva. The interaction of GAS with saliva is crucial for the establishment of pharyngitis and the subsequent transmission of infectious organisms (19, 20, 26, 46). Although human saliva is known to support the growth of oral streptococci, little is known about molecular processes contributing to the growth of GAS in this fluid (4, 8, 9, 42). We established that GAS grew to a density of ~10⁷ CFU/ml of saliva, a finding that is very similar to that observed for *Strep-*

FIG. 5. Western immunoblots showing production of GAS extracellular proteins during growth in saliva. GAS strains were grown in saliva medium B for 4 h, 8 h, and 16 h (corresponding to early exponential, late exponential, and early stationary phase). Proteins present in the supernatant were precipitated, separated by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis, and transferred to nitrocellulose. The membranes were probed with primary rabbit antibody (1:10,000) specific for each protein and secondary antibody (1:2,500) conjugated to horseradish peroxidase. Reactivity was visualized with chemiluminescent reagents. Saliva without GAS added was used as a negative control, and the purified protein of interest served as a positive control. Sic was assayed during growth of serotype M1 strain MGAS5005; SlaA was assayed during growth of serotype M3 strain MGAS315; SpeB was assayed during growth of serotype M1 strain MGAS5005 at 4, 8, and 16 h as well as at 7 days and 28 days. The degradation of Sic observed at 16 h has been reported previously (15). N.D., not done.

tococcus gordonii (42). The ability of GAS to grow in human saliva to this density is consistent with reported densities of 5×10^6 organisms per milliliter of saliva in patients with symptomatic GAS pharyngitis (19, 26, 46).

The factors limiting the growth of GAS in human saliva in vitro and in vivo are not understood. It has been suggested that the level of free glucose in saliva is not sufficient to support cell division of oral streptococci to values exceeding 1×10^7 CFU/ml (42). However, similar to other investigators, we found that the addition of glucose did not increase the density of organisms, suggesting that the concentration of glucose

alone is not likely to be a limiting factor for growth (9). We also found that pH or the addition of DTT did not affect the maximum growth achieved in vitro.

The ability of human saliva to restrict the density of microorganisms such as GAS to $\sim 10^7$ CFU/ml is in striking contrast to the growth-promoting ability of other body fluids. Bacteria growing in blood, urine, or cerebrospinal fluid can reach densities of 10^9 CFU/ml and higher (7, 29, 45). The presence of multiple antimicrobial peptides and the limited nutrients available in saliva for the growth of microorganisms likely contribute to this phenomenon (1, 25). The observation that saliva limits microbial proliferation confirms the fundamental contribution of saliva to innate immune defense of the human oropharynx.

The persistence in saliva for at least 28 days at near-maximal growth levels of all serotype M1 strains studied was very unexpected (Fig. 2). This persistence contrasted with our findings, also observed by other investigators (53), that in nutrient-rich media (THY) and in nutrient-limited media (CDM), GAS had a rapid decrease in viable organisms after reaching stationary phase. We speculate that when a cell density of $\sim 1 \times 10^7$ CFU/ml is reached in saliva, GAS enters a state of restricted metabolic activity, thereby allowing persistence at a relatively high density, a phenomenon which would be expected to enhance the likelihood of transmission to a new host. The idea of restricted metabolic activity (and presumably decreased cell wall turnover) is supported by the observation that during the stationary phase in saliva, GAS had decreased susceptibility to killing by ampicillin.

Growth and persistence of GAS strains in saliva. Although there is significant geographic variation in the relative frequency of pharyngitis caused by distinct M-protein serotypes, several M types tend to predominate in many areas (21, 49). For example, serotype M1 strains are the leading cause of GAS pharyngitis in many studies (11, 49). Thus, it is noteworthy that

FIG. 6. Growth of GAS *sic* and *speB* isogenic mutant strains in saliva. Growth was assayed by removing aliquots from saliva medium B at indicated time points and counting CFU after overnight incubation on BSA at 37°C in 5% CO₂. Recombinant Sic and SpeB were added to culture medium as described in Materials and Methods to assess complementation. Symbols: wild-type serotype M1 strain MGAS5005, ■, solid line; isogenic *sic* mutant strains, ▼, dashed line; isogenic *sic* mutant strain with recombinant Sic, ◆, solid line; isogenic *speB* mutant strain, ▲, dashed line; isogenic *speB* mutant strain with recombinant SpeB, □, solid line. Bars represent standard deviations.

TABLE 2. Growth of isogenic mutant GAS strains in saliva^a

Name or SPy no. ⁱ of mutated gene	Gene product ^b	Maximum cell density ^c	<i>P</i> value ^d	Cell density at 168 h ^e	<i>P</i> value ^d	Reference
Wild-type MGAS5005	NA ^h	6.87 ± 0.07	NA	6.49 ± 0.07	NA	35
<i>sic</i>	Streptococcal inhibitor of complement	6.02 ± 0.07	<0.001	5.58 ± 0.16	<0.001	23
<i>speB</i>	Extracellular cysteine protease	6.91 ± 0.10	1.00	4.66 ± 0.18	<0.001	38
<i>mac</i>	Immune modulating protein	6.84 ± 0.11	1.00	6.56 ± 0.13	1.00	30
<i>SlaA</i> ^f	Streptococcal phospholipase A ₂	7.13 ± 0.21	1.00	6.08 ± 0.12	1.00	41
SPy2191	Extracellular protein	6.95 ± 0.05	1.00	6.53 ± 0.10	1.00	57
<i>covR</i> (<i>csrR</i>)	TCS response regulator	6.92 ± 0.04	1.00	6.71 ± 0.07	0.420	17
<i>ihk</i> ^g	TCS response regulator	7.25 ± 0.08	1.00	5.34 ± .30	1.00	57
SPy1062	TCS response regulator	6.76 ± 0.16	1.00	6.65 ± 0.03	0.781	44
SPy1106	TCS response regulator	6.67 ± 0.14	1.00	6.57 ± 0.09	1.00	44
SPy1198	Repressor protein	6.43 ± 0.10	0.10	6.54 ± 0.16	1.00	This study
SPy1556	TCS response regulator	6.83 ± 0.16	1.00	6.52 ± 0.11	1.00	44
SPy1587	TCS response regulator	6.72 ± 0.09	1.00	6.52 ± 0.08	1.00	17
SPy1634	Transcriptional regulator	6.69 ± 0.08	1.00	6.48 ± 0.07	1.00	This study
SPy1960	Transcriptional regulator	6.73 ± 0.10	1.00	6.56 ± 0.08	1.00	This study
SPy2053	Transcriptional regulator	6.57 ± 0.18	1.00	6.11 ± 0.19	1.00	This study
SPy2074	Transcriptional regulator	6.63 ± 0.18	1.00	6.74 ± 0.07	0.51	This paper

^a Growth was assayed in saliva medium B; numbers of CFU were determined by plating serial dilutions.^b TCS, two-component system.^c Highest density (log₁₀ CFU/ml) achieved during incubation.^d *P* value for comparison with wild-type strain as determined by paired Student's *t*-test with Bonferroni's adjustment for multiple comparisons.^e Log₁₀ CFU/ml, determined at 168 h.^f *SlaA* mutant strain was compared with its parent strain, MGAS315.^g *ihk* mutant strain was compared with its parent strain, JRS4.^h NA, not applicable.ⁱ SPy no., *Streptococcus pyogenes* gene number.

we found that serotype M1 strains persisted in saliva at higher densities and for longer periods than did the strains of all other serotypes studied. We speculate that the persistence of serotype M1 strains in human saliva contributes to the prevalent nature of serotype M1 strains as a cause of pharyngitis. It is

possible that the production of Sic by these strains is an important factor in this regard (see below).

Overall, we did not observe a simple relationship between the magnitude of persistence in saliva and reported abundance of a GAS serotype in human infections. For example, strains of

TABLE 3. Extracellular protein production by GAS strains during growth in human saliva^a

Protein	Function	Strain assayed (M serotype)	Presence of immunoreactive material		
			4 h	8 h	16 h
Sic	Inhibitor of phagocytosis and killing	MGAS5005 (M1)	+	+	+/-
Mac	Inhibitor of opsonophagocytosis	MGAS5005 (M1)	+	+	+
		MGAS315 (M3)	-	+	+
		MGAS6180 (M28)	-	+	+
SpeA	Superantigen (scarlet fever toxin)	MGAS5005 (M1)	-	+	+
		MGAS315 (M3)	-	+	+
SpeB	Secreted cysteine protease	MGAS5005 (M1)	-	-	+
		MGAS315 (M3)	-	+	+
		JRS4 (M6)	ND	ND	-
		MGAS9645 (M6)	ND	ND	+
		MGAS10287 (M6)	ND	ND	+
		MGAS12487 (M9)	ND	ND	+
		MGAS12292 (M11)	ND	ND	+
		MGAS9429 (M12)	ND	ND	+
		MGAS6180 (M28)	-	-	+
		MGAS9530 (M33)	ND	ND	+
		MGAS9464 (M43)	ND	ND	-
		MGAS12083 (M73)	ND	ND	+
		MGAS9482 (M77)	ND	ND	+
		MGAS12349 (M94)	ND	ND	-
SlaA	Secreted phospholipase A ₂	MGAS315 (M3)	+	+	+
		MGAS6180 (M28)	+	+	+
SPy2191	Unknown	MGAS5005 (M1)	-	+	+
		MGAS315 (M3)	+	+	+
		MGAS6180 (M28)	-	+	+

^a Presence of immunoreactive material assayed by Western immunoblotting. +, reactivity detected; -, reactivity not detected; +/-, indeterminate; ND, not done.

serotypes that rarely cause pharyngitis, such as M9, M11, and M33, persisted at levels similar to those of strains of serotypes that often cause pharyngitis. The difficulty of using individual strains to generally characterize a serotype is illustrated by our results with the M6 serotype strains, where there were marked differences in the abilities of individual strains to persist in saliva. Taken together, our results suggest that the growth and persistence of a GAS strain of a particular serotype in human saliva are not alone sufficient to account for that serotype's relative abundance in pharyngitis cases, although they may contribute. It will be important to investigate this issue with a larger sample of GAS strains.

The role of Sic and SpeB in the growth of GAS in human saliva. GAS elaborates at least two extracellular proteins, SpeB and Sic, that have been shown to inhibit or inactivate the purified forms of antimicrobial peptides present in saliva (13, 14, 16, 47). Sic is produced mainly by serotype M1 strains, whereas virtually all strains have the *speB* gene, although protein expression levels are variable (16, 27, 52). Sic appears to function through binding inhibition, whereas the cysteine protease SpeB can actively degrade peptides involved in innate immunity (14, 47). We tested the hypothesis that the activities of SpeB and Sic contributed to the ability of GAS to proliferate in saliva, using isogenic mutant strains in which SpeB and Sic were not produced. Both the *sic* and *speB* isogenic mutant strains had decreased growth in human saliva, and this deficiency was reversed by the addition of the respective purified protein to the culture medium. Moreover, GAS strains that failed to persist in saliva did not produce detectable amounts of SpeB during culture in saliva. Inactivation of Sic has previously been shown to lead to a decreased ability of GAS to colonize the mouse oropharynx (35). Our results suggest that the failure of the *sic* mutant strain to successfully colonize the oropharynx may have been due in part to its inability to persist at high levels in saliva. Taken together, we conclude that Sic and SpeB are crucial to a successful GAS interaction with human saliva.

GAS extracellular protein production during growth in saliva. Having established a role for Sic and SpeB for GAS-saliva interaction, we next sought to determine if GAS secreted other extracellular proteins during growth in saliva. We found that several proven and putative extracellular virulence proteins (e.g., SpeA, Mac, SlaA, and SPy2191) were produced during growth in human saliva (Table 2). Moreover, these proteins were produced in a growth-phase-dependent fashion, suggesting that transcriptional regulation of GAS virulence factor production occurred during growth in saliva.

Taken altogether, our results shed new light on a key early event in the life cycle of GAS, namely, the interaction between GAS and human saliva. Recent reports have documented up-regulation of several GAS genes in response to contact with human epithelial cells in vitro, another important event in the life cycle of this pathogen (2, 5, 6, 41). However, by showing that GAS responded in a complex fashion to growth in human saliva, our results suggest that molecular processes that enhance colonization and survival in the upper respiratory tract of humans are well under way before the organism reaches the epithelial cell surface. Thus, further study of GAS-saliva interaction is likely to provide new insights into the molecular strategies used by this common human pathogen to success-

fully colonize the human oropharynx, produce disease, and disseminate to a new host.

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