Biofilm formation by group A *Streptococcus*: a role for the streptococcal regulator of virulence (Srv) and streptococcal cysteine protease (SpeB)

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Recently, biofilms have become a topic of interest in the study of the human pathogen group A *Streptococcus* (GAS). In this study, we sought to learn more about the make-up of these structures and gain insight into biofilm regulation. Enzymic studies indicated that biofilm formation by GAS strain MGAS5005 required an extracellular protein and DNA component(s). Previous results indicated that inactivation of the transcriptional regulator Srv in MGAS5005 resulted in a significant decrease in virulence. Here, inactivation of Srv also resulted in a significant decrease in biofilm formation under both static and flow conditions. Given that production of the extracellular cysteine protease SpeB is increased in the *srv* mutant, we tested the hypothesis that increased levels of active SpeB may be responsible for the reduction in biofilm formation. Western immunoblot analysis indicated that SpeB was absent from MGAS5005 biofilms.

Complementation of MGAS5005 Δ *srv* restored the biofilm phenotype and eliminated the overproduction of active SpeB. Inhibition of SpeB with E64 also restored the MGAS5005 Δ *srv* biofilm to wild-type levels.

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INTRODUCTION

Group A *Streptococcus* (GAS) is responsible for a wide range of human infections, including pharyngitis and tonsillitis, skin infections, sepsis, a toxic-shock syndrome, and necrotizing fasciitis (Cunningham, 2000; Musser & Krause, 1998; Reid *et al.*, 2001). Until recently, there was little association of GAS and biofilms in the published literature. One of the first observations was reported by Akiyama *et al.* (2003) in a study examining GAS in skin infections. Confocal laser scanning microscopy of skin lesions revealed the presence of GAS microcolonies that appeared to be surrounded by a glycocalyx (Akiyama *et al.*, 2003). A limited number of subsequent studies have demonstrated GAS biofilm formation *in vitro* and *in vivo* and microarray analyses have shown that gene transcription varies markedly in biofilms compared to planktoni-

cally grown GAS (Baldassarri *et al.*, 2006; Cho & Caparon, 2005; Conley *et al.*, 2003; Lembke *et al.*, 2006; Manetti *et al.*, 2007; Riani *et al.*, 2007; Takemura *et al.*, 2004). However, little is known about whether the ability to form biofilms is a characteristic of all GAS strains, what the biofilm matrix is composed of, or how this biofilm state is regulated.

In the present work, we demonstrate that a globally disseminated M1T1 serotype clone associated with invasive disease is capable of producing a structured, surface-attached community resembling a biofilm. In addition, we demonstrate that DNA and protein are critical to the formation of this structure. Furthermore, in an effort to gain insight into the regulation of the biofilm process, we examined the effects of loss of the virulence regulator Srv on GAS biofilm formation. Srv has been shown to be required for GAS virulence and to influence GAS gene expression (Reid *et al.*, 2004, 2006). Here we show that Srv is required for biofilm formation and that increased activity of the cysteine protease SpeB in the absence of Srv contributes to the loss of the biofilm phenotype.

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Abbreviations: CV, crystal violet; GAS, group A *Streptococcus*.

METHODS

Bacterial strains. MGAS5005 is a representative M1T1 serotype strain (*speA*⁺) which was isolated from a case of invasive GAS disease and has been the focus of numerous studies (reviewed by Musser & Krause, 1998; Reid *et al.*, 2001). The MGAS5005 isogenic *srv* mutant strain (MGAS5005Δ*srv*) was generated by allelic replacement (Reid *et al.*, 2004). MGAS5005Δ*srv* was complemented *in trans* [MGAS5005Δ*srv*(pIAβ8-*srv*)] as previously described (Doern *et al.*, 2008).

Adherence assay. Overnight cultures of GAS grown at 37 °C (5 % CO₂) in Todd–Hewitt Broth (TH) (Difco) supplemented with 0.2 % yeast extract (THY) (Fisher Scientific) were harvested and used to inoculate fresh THY. Cultures were then grown to an OD₆₀₀ of 0.5. Tissue culture treated polystyrene six-well cell culture plates (Corning) were seeded with 3 ml of culture per well. Plates were incubated for 24 h at 37 °C, 5 % CO₂. Medium was removed without disturbing the biofilm and wells were washed three times with distilled H₂O (dH₂O). Then 1 ml aliquots of 0.1 % crystal violet (CV) (Sigma-Aldrich) dissolved in dH₂O were dispensed to each well. Surface-attached bacteria were allowed to stain for 30 min at room temperature and then washed three times with dH₂O, after which 1 ml ethanol was added to each well to solubilize the CV. An A₆₀₀ reading (measuring the colour intensity of the solubilized CV) was recorded for each sample. If staining was too dark for spectrophotometric analysis, the sample was diluted with additional ethanol and the dilution factor applied to the resulting reading (in these instances, the value is referred to as the A₆₀₀ extrapolated, or A_{600e}).

Enzymic inhibition/disruption of biofilms. Methods were adapted from Wang *et al.* (2004). To test the ability to inhibit biofilm formation, 2 ml aliquots of exponentially growing GAS cultures (OD₆₀₀ 0.5) were mixed with 2 ml of one of the following reagents (final concn): 40 mM NaIO₄ (metaperiodate), 200 μg DNase I ml⁻¹ or 1 mg proteinase K ml⁻¹. Enzyme-treated cultures were added to six-well tissue culture plates and incubated for 24 h at 37 °C, 5 % CO₂. Wells were washed and stained with CV as described above. To test the ability to disrupt a pre-formed biofilm, each of the reagents described above was added to a 24-h-old biofilm and allowed to incubate for an additional 1 h. Wells were washed and stained as before.

Analysis of biofilm formation under continuous-flow conditions. Using a 25 gauge 1½ inch Precision Glide needle (Becton Dickinson), a 10 ml aliquot of exponentially growing GAS culture (OD₆₀₀ 0.5) was inoculated into a convertible flow-cell chamber (Stovall Life Sciences). Each inoculum was incubated for 3 h at 37 °C without flow to allow initial attachment within the chamber. Sterile THY medium was connected to the flow chamber through the pump head via Masterflex silicon tubing (peroxide treated) L/S14 (Cole-Parmer Instruments), and flow discharge was collected in a waste container downstream of the flow-cell chamber. Flow was initiated using a Masterflex Easy-Load II peristaltic pump (Cole-Parmer Instruments) with a flow rate of 0.7 ml min⁻¹ and continued for 24 h at 37 °C.

Confocal laser scanning microscopy of flow-cell biofilms. To visually examine the health of the biofilm, samples were subjected to live/dead staining. Samples contained within flow-cell chambers were washed with PBS. A mixture of SYTO 9 and propidium iodide (LIVE/DEAD BacLight Bacterial Viability kit L7007; Molecular Probes) contained in 10 ml PBS was added to the chamber. The sample was allowed to incubate at room temperature for 1 h with the LIVE/DEAD stain and then washed with PBS. Samples were visualized using a Zeiss LSM510 confocal scanning laser microscope.

Scanning electron microscopy of flow-cell biofilms. Continuous flow-cell samples were fixed within the flow-cell chamber with 2.5 % glutaraldehyde in PBS. After fixation for a minimum of 1 h, samples were washed twice and subjected to dehydration, fixation, and critical-point drying for scanning electron microscopy. Flow-cell chamber slides were then trimmed, mounted, and coated with palladium. Biofilms were viewed on a Phillips SEM-515 scanning electron microscope.

RESULTS

Protein and DNA are significant components of the GAS biofilm

We first determined the level of biofilm formed by MGAS5005, a representative serotype M1T1 strain recently isolated from a case of invasive GAS disease. Population genetic analysis has indicated that M1T1 strains are among the most common causes of invasive GAS infections worldwide in most case studies (Musser & Krause, 1998; Reid *et al.*, 2001). Dilution of the solubilized CV was necessary as the original CV staining was too dark for spectrophotometric analysis.

We next sought to learn more about the biofilm structure by examining the components present. We hypothesized that if proteins, DNA or polysaccharides are required, then biofilms should be inhibited or disrupted by proteinase K, DNase I, or metaperiodate, respectively. Addition of proteinase K or DNase I at the time six-well plates were seeded significantly inhibited biofilm formation (Fig. 1a). In addition, a 1 h incubation with either proteinase K or DNase I disrupted an existing 24 h old biofilm (Fig. 1b). Treatment with metaperiodate, a compound capable of oxidizing polysaccharides (Mack *et al.*, 1996; Maira-Litran *et al.*, 2002; Wang *et al.*, 2004), led to a reduction in both the formation and the stability of the biofilm, but measurable biofilm was still present (Fig. 1a, b). As a control, we performed replicate plating to test the viability of the bacteria following treatment. C.f.u. numbers recovered following treatment with proteinase K or DNase I were equivalent to those following treatment with PBS (~10⁹ c.f.u. ml⁻¹). However, we recovered 10⁶–10⁷ c.f.u. ml⁻¹ from metaperiodate-treated samples. This suggested that the decrease in biofilm observed may be due to the effects of metaperiodate on bacterial viability. Thus, the GAS biofilm requires a protein and a DNA component(s) for formation and stability. GAS polysaccharides may increase the mass of the biofilm when present, but periodate-sensitive polysaccharides do not appear to be required for GAS biofilm formation.

Inactivation of the transcriptional regulator Srv results in a significant reduction in GAS biofilm formation

The observation that extracellular protein is important in the formation of the GAS biofilm led us to consider the possible role of the transcriptional regulator Srv. Our previous studies indicated that inactivation of *srv* resulted

Fig. 1. Enzymic inhibition or disruption of GAS biofilm formation. (a) To test the ability of specific enzymes to inhibit biofilm formation, proteinase K, DNase I or metaperiodate was added when the six-well plates were initially seeded with MGAS5005 in the exponential phase (OD_{600} 0.5). After 24 h growth, strains incubated with proteinase K or DNase I produced significantly less, if any, biofilm. While metaperiodate curtailed biofilm formation, it did not abolish this ability. Each reported value is the mean $\pm$ SD of at least six replicates and is adjusted by the dilution factor required to obtain a spectrophotometric reading (A_{600} extrapolated or A_{600e}). (b) To test the ability of the same enzymes to disrupt an established GAS biofilm, proteinase K, DNase I or metaperiodate was added to cultures grown in six-well plates for 24 h. CV assays were completed after 1 h incubation with enzyme. Treatment with proteinase K or DNase I disrupted the biofilm and yielded a significantly reduced A_{600e} . Treatment with metaperiodate reduced the amount of biofilm present, but was unable to completely disrupt the biofilm.

in a significant reduction in GAS virulence (Reid *et al.*, 2004) and differential transcription of 51 genes encoding proven or putative extracellular proteins (Reid *et al.*, 2006). The reduced expression of genes encoding extracellular proteins in the *srv* mutant strain led us to hypothesize that Srv may contribute to biofilm formation. Comparison of MGAS5005 and the *srv* isogenic mutant derivative MGAS5005 Δ *srv* indicated that MGAS5005 Δ *srv* produced significantly less biofilm (Fig. 2a). Complementation of *srv* *in trans* [MGAS5005 Δ *srv*(pIA β 8-*srv*)] restored biofilm production to near wild-type levels (Fig. 2a).

To determine if the results observed were due to an impaired ability of MGAS5005 Δ *srv* to form biofilms or an early disruption of the biofilm, we followed formation of the structure over time. MGAS5005 and MGAS5005 Δ *srv*(pIA β 8-*srv*) produced biofilm at approximately the same rate and to the same level (Fig. 2b). Maximal biofilm formation was observed at 24 h, with levels tapering off thereafter. In contrast, CV staining of surface-attached MGAS5005 Δ *srv* was significantly less at all time points measured (Fig. 2b).

Inhibition of SpeB in MGAS5005 Δ *srv* restores biofilm formation

Our data suggested that Srv-mediated gene transcription was required for proper biofilm formation. This may be

Fig. 2. The transcriptional regulator Srv is required for maximal biofilm formation by MGAS5005. Each reported value for the CV assay is an average of at least six replicates and is adjusted by the dilution factor required to obtain a spectrophotometric reading (A_{600e}). (a) A comparison of the wild-type (MGAS5005), the isogenic mutant lacking *srv* (Δ *srv*), and the complemented strain [MGAS5005 Δ *srv*(pIA β 8-*srv*)]. The Δ *srv* mutant is significantly reduced in its ability to form biofilms. Complementation of *srv* *in trans* restores biofilm formation. The mean A_{600} (extrapolation unnecessary) for wells containing THY alone is provided as a negative control. (b) Kinetics of GAS biofilm formation. The A_{600e} values of solubilized CV from surface-attached cells grown in six-well polystyrene plates were assayed over time.

due to reduced transcription of genes encoding extracellular proteins required for attachment and/or aggregation, or to the effects of some other product that is directly or indirectly affected by Srv. Previously, we discovered that inactivation of *srv* led to increased production of the GAS cysteine protease SpeB (Reid *et al.*, 2006). We can visualize the increased activity of SpeB in a casein agar assay, in which strains are stab inoculated into casein agar and allowed to grow at 37 °C under microaerophilic conditions. The resulting zone of translucence surrounding the stab site is indicative of SpeB caseinolytic activity. This assay demonstrated that complementation with *srv* *in trans* restored SpeB activity to wild-type levels (Fig. 3).

To test the hypothesis that an increase in SpeB may be responsible for the MGAS5005 Δ *srv* biofilm defect, we

Fig. 3. Casein agar assay for SpeB proteolytic activity. MGAS5005, Δsrv and MGAS5005 Δsrv (pIA β 8-*srv*) were stab inoculated into agar plates containing skim milk and incubated for 18 h microaerophilically. Protease activity is manifest as a zone of translucence surrounding the stab sites. There is a marked increase in the amount of SpeB proteolysis exhibited by Δsrv compared to MGAS5005 or MGAS5005 Δsrv (pIA β 8-*srv*).

examined biofilm formation in the presence of an inhibitor of SpeB activity, E64. E64 is a potent and highly selective cysteine protease inhibitor that irreversibly binds to an active thiol group to form a thioether linkage. Previous groups have used concentrations of ~30 μ M to 10 mM E64 (Kansal *et al.*, 2003; Vincents *et al.*, 2004). However, in these studies the inhibitor was added to assays using GAS supernatants or purified proteins. Since we wished to add E64 to living cells and allow the culture to incubate for 24 h, we elected to use a final concentration of 333 μ M. Addition of E64 at the time the plates were seeded restored the ability of MGAS5005 Δsrv to form biofilms (Fig. 4a). To determine that the observed results were due to the inhibition of SpeB and not another protease, we examined the biofilm formation of a *speB* isogenic mutant (Lukomski *et al.*, 1998) with and without the addition of E64 (Fig. 4a). No difference was observed in the ability of the $\Delta speB$ strain to form biofilms. Examination of the attached bacteria prior to solubilization with ethanol revealed that the CV stain was not as uniform in E64-treated MGAS5005 Δsrv as in the treated wild-type, but there was a clear increase in attached bacteria compared to untreated MGAS5005 Δsrv (Fig. 4b).

Extracellular SpeB is absent from 24 h GAS biofilms

Previously, we demonstrated that SpeB was detected by Western immunoblotting in culture supernatants of MGAS5005 following 8 h of planktonic growth (Reid *et al.*, 2006). By contrast, SpeB was detectable in MGAS5005 Δsrv supernatants after just 2 h of growth (Reid *et al.*, 2006). For the experiments presented above, cultures were allowed to grow planktonically for 3 h prior to seeding of the six-well plates. Biofilms were then allowed to develop for 24 h. If SpeB inhibits or disperses the GAS biofilm, then it must be present at very low levels if at all in

Fig. 4. Inhibition of SpeB activity restores GAS biofilm formation. (a) Graphical representation of CV assays of biofilm-forming ability in the presence and absence of the cysteine protease inhibitor E64. While addition of E64 may reduce the biofilm capacity of the wild-type slightly, biofilm formation of the Δsrv strain is restored to comparable levels. E64 did not alter biofilm formation by the $\Delta speB$ isogenic mutant strain, suggesting that the alteration in biofilm phenotype is due to SpeB activity. The data are means $\pm$ SD of at least six replicates. (b) Single-well images of CV assays prior to solubilization in ethanol. After image capture, the CV from surface-attached cells was solubilized and the A_{600e} values are shown. Note the increase in stained material in the Δsrv well in the presence of E64.

the biofilm. To test this hypothesis, GAS biofilms and their supernatants were collected, treated as a single sample (biofilm and supernatant together), and probed by Western immunoblot analysis. Abundant SpeB was detected in the sample from the biofilm-deficient MGAS5005 Δsrv (Fig. 5). However, SpeB was undetectable in MGAS5005 samples, and only trace amounts of SpeB-zymogen (inactive) were detected in MGAS5005 Δsrv (pIA β 8-*srv*) samples (Fig. 5). As a negative control, samples from MGAS5005 $\Delta speB$ were probed (Fig. 5) (Lukomski *et al.*, 1998).

Analysis of GAS biofilm formation using continuous flow

Research has indicated that the growth and structures of bacterial biofilms are affected by hydrodynamic shear that may be generated by the flow of fluids over epithelial surfaces in the host (Lappin-Scott & Bass, 2001; Purevdorj *et al.*, 2002; Stoodley *et al.*, 2001). While our data indicate that GAS is capable of forming dense, surface-attached communities under static conditions, we sought to determine the ability of GAS to form biofilms under continuous flow. The convertible flow-cell chamber was

Fig. 5. Detection of SpeB in GAS biofilms. Western immunoblot analysis failed to detect the presence of SpeB in supernatants collected from MGAS5005 and MGAS5005Δ*speB* after growth for 24 h in a biofilm state. Elevated levels of SpeB were detected in supernatants collected from MGAS5005Δ*srv*. Low levels of the inactive form of SpeB (zymogen) were detected in supernatants collected from the *srv*-complemented strain MGAS5005Δ*srv*(pIAβ8-*srv*).

inoculated with 10 ml of the wild-type, the *srv* mutant or the complemented strain, harvested at OD₆₀₀ 0.5, and the bacteria were allowed to attach for 3 h at 37 °C. Flow of

fresh THY medium was then initiated at a rate of ~0.7 ml min⁻¹, and the chamber was incubated for an additional 24 h. After 24 h of growth, the MGAS5005 and the MGAS5005Δ*srv*(pIAβ8-*srv*) flow chambers were completely filled with a viscous material (Fig. 6a). Plating of a sample of this material revealed GAS and no contaminants (data not shown). As observed under static conditions, MGAS5005Δ*srv* was largely unable to form a biofilm (Fig. 6a). Small pockets of attachment were observed for MGAS5005Δ*srv*, but these pockets were washed away when the chamber was flushed with PBS in preparation for microscopic analyses. Taken together, these data and our analysis of static biofilms over time (Fig. 2b) suggested that MGAS5005Δ*srv* was deficient in attachment.

To determine the viability of cells within the biofilm, the MGAS5005 within the flow-cell was stained with a commercial reagent (LIVE/DEAD, Molecular Probes) and visualized by confocal microscopy (Fig. 6b). By this method, living cells with intact membranes are stained green with SYTO 9 while dead cells are stained red with propidium iodide. Colocalization of both signals gives a yellow colour. After 24 h, the majority of cells in the biofilm were alive. Fifty consecutive micrographs of an X–Y plane indicated that the biofilm was approximately 80 μm thick. Vertical stacked Z images (margins of Fig. 6b) showed living cells dispersed throughout the macroscopic structure.

Electron microscopy revealed a densely packed population of MGAS5005 that appeared to be encased in an extracellular matrix (Fig. 6c, d). An angled image of the biofilm removed from the corner of the flow chamber revealed the three-dimensional nature of the structure

Fig. 6. Continuous-flow system for study of GAS biofilm formation. (a) Inactivation of *srv* eliminates the ability of GAS to form biofilms under continuous-flow conditions. Complementation of *srv* in trans [MGAS5005Δ*srv*(pIAβ8-*srv*)] restores the biofilm phenotype. (b) Confocal microscopy of a 24 h continuous-flow biofilm. The image is an X–Y micrograph of MGAS5005 biofilm stained with LIVE/DEAD reagent. Cells with intact membranes (living) are stained green while cells with disrupted membranes are stained red. Areas of overlap appear yellow. Fifty X–Y plane micrographs indicate that the biofilm is approximately 80 μm thick. Vertical stacked Z images (margins) show living cells dispersed throughout the macroscopic structure. (c, d) Scanning electron microscopy of a 24 h continuous-flow biofilm; (c) shows the thickness of the biofilm formed within the chamber. At high magnification (d), chains of GAS can be clearly seen. Note the matrix material that appears to cover the bacterial chains. White arrows indicate the presence of fibrous strands.

(Fig. 6c). Higher magnification allowed the visualization of chains of bacteria which appeared to be coated in matrix (Fig. 6d). Fibrous strands could be seen running within the matrix (Fig. 6d, white arrows). Some of these appeared to be sheared, probably during the fixation process.

DISCUSSION

In a recent study, Baldassarri *et al.* (2006) examined a total of 289 GAS isolates from different clinical sources and demonstrated that 90 % of those strains were capable of forming biofilms. In this investigation, we sought to learn more about the make-up of these structures and gain insight into biofilm regulation. Enzymic treatment of GAS biofilms demonstrated a requirement for DNA and protein in the structure, but suggested only a passive role for carbohydrate. This is in contrast to numerous systems, for example *Pseudomonas aeruginosa*, for which several genetically encoded polysaccharides have been shown to be required (Donlan, 2001; Donlan & Costerton, 2002; Hall-Stoodley *et al.*, 2004; Ryder *et al.*, 2007). Cho & Caparon (2005) found that the GAS hyaluronic acid capsule (HasA) was not required for biofilm formation in a static system (CV assay), but that following initial attachment, the *hasA* mutant was unable to aggregate under flow conditions. Given the volume of enzyme that would be required, we were unable to test inhibition or disruption under flow conditions. Thus, hyaluronic acid does not appear necessary for initial biofilm attachment, but it may be needed for aggregation and biofilm maturation. Alternatively, differences between the serotype M1 strain used here and the Cho M5 strain may account for the observed differences. Further assessment *in vivo* will address these possibilities.

Increasing evidence suggests not only that DNA is a major component of bacterial biofilms, but that genetically encoded systems controlling programmed cell death provide a mechanism for the release of bacterial DNA (reviewed by Bayles, 2007). Therefore, it is not surprising that we demonstrated a role for DNA in GAS biofilms. However, the process by which GAS releases DNA is not yet known. GAS does not appear to encode *cid/lrg* homologues which control programmed cell death in other bacterial species, although the presence of additional or diverged genes encoding holins cannot be ruled out.

DNA, or more appropriately the lack of extracellular DNA, has been linked to severe GAS infection. Evidence has been presented that MGAS5005 has a mutation within the GAS control of virulence operon (*covRS/csrRS*) which limits or abolishes *speB* expression. This is hypothesized to allow the production of a GAS extracellular DNase (Sda1), a protein that would normally be degraded by SpeB (Buchanan *et al.*, 2006; Sumby *et al.*, 2005; Walker *et al.*, 2007). According to this model, Sda1 facilitates the escape from neutrophil extracellular traps (NETs) by degrading the associated neutrophil DNA (Brinkmann *et al.*, 2004; Walker *et al.*,

2007). The infecting strain is then free to disseminate and potentially cause severe invasive disease (Sumby *et al.*, 2006; Walker *et al.*, 2007). Our data indicate that DNA is required for MGAS5005 biofilm formation *in vitro*. This suggests that production of Sda1 is not solely dependent on the absence of SpeB, but that it requires a signal encountered *in vivo*. This idea is in concert with the data of Sumby *et al.* (2006), who found naturally occurring examples of the *covS* mutation following passage in a mouse model.

It is of note that our data also indicate that inactivation of *srv* in MGAS5005 compensates for the *covS* mutation and restores SpeB production in this strain. In addition, our data indicate that there is a necessary protein component for initial adherence and/or aggregation leading to biofilm maturation. We hypothesized that the high levels of SpeB may be responsible for the biofilm-deficient phenotype of the *srv* mutant. Addition of the cysteine protease inhibitor E64 restored the ability of MGAS5005Δ*srv* to form biofilms, suggesting that SpeB degrades GAS proteins needed for establishment of the biofilm (E64-treated MGAS5005Δ*speB* produced wild-type levels of biofilm). This leads to the hypothesis that the timed production of SpeB might contribute to GAS biofilm dispersion. Under this model, GAS begins in a biofilm state. In reaction to an unknown stimulus, an alteration in Srv-mediated control occurs and results in the direct or indirect increase of SpeB production and/or secretion. SpeB degrades GAS and host proteins integral to the biofilm. This alone may disperse the biofilm or, as put forth by others, the increased activity of and resulting damage from SpeB is perceived as a signal (in addition to host signals) which drives selection for mutations in *covS* (Sumby *et al.*, 2006; Walker *et al.*, 2007). Inactive CovS leads to the repression of *speB*, induction of *sdaI*, and the degradation of host and bacterial DNA (which may be more accessible due to the prior cleavage of proteins by SpeB). GAS bacteria are now free to disperse and potentially adopt an invasive phenotype (Sumby *et al.*, 2006; Walker *et al.*, 2007). While this model is preliminary, we believe it is testable. Clearly, further study of GAS biofilm formation will provide new insights into the pathogenesis of GAS.

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