Role for sagA and siaA in Quorum Sensing and Iron Regulation in Streptococcus pyogenes

Kowthar Y. Salim, Joyce C. de Azavedo, Darrin J. Bast, and Dennis G. Cvitkovitch

Department of Microbiology, Dental Research Institute, University of Toronto, Ontario, Canada, and Department of Laboratory Medicine and Pathobiology, University of Toronto, Ontario, Canada

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Streptococcus pyogenes is a ubiquitous and versatile pathogen that causes a variety of infections with a wide range of severity. The versatility of this organism is due in part to its capacity to regulate virulence gene expression in response to the many environments that it encounters during an infection. We analyzed the expression of two potential virulence factors, sagA and siaA (also referred to as pel and htsA, respectively), in response to conditions of varying cell densities and iron concentrations. The sagA gene was up-regulated in conditioned medium from a wild-type strain but not from siaA-deficient mutants, and the gene was also up-regulated in the presence of streptolysin S (SLS), the gene product of sagA, thus indicating that this gene or its product is involved in density-dependent regulation of S. pyogenes. By comparison, siaA responded in a manner consistent with a role in iron acquisition since it was up-regulated under iron-restricted conditions. Although siaA expression was also up-regulated in the presence of SLS and in conditioned media from both wild-type and sagA-deficient mutants, this up-regulation was not growth phase dependent. We conclude that sagA encodes a quorum-sensing signaling molecule, likely SLS, and further support the notion that siaA is likely involved in iron acquisition.

Streptococcus pyogenes is responsible for a variety of human diseases occurring at different body sites, such as pharyngitis, cellulitis, impetigo, necrotizing fasciitis, and streptococcal toxic shock syndrome. Multiple virulence factors enable this organism to colonize, evade immune defenses, and spread within the human host. These processes must be precisely regulated in order for S. pyogenes to survive and replicate within the numerous environmental challenges it encounters during infection. Consequently, the regulation of its gene expression is complex and involves an array of interacting regulators. Two such environmental challenges encountered by S. pyogenes during infection include high cell density, which can occur during tissue infections, and a lack of freely available iron within the human host.

Iron is an essential nutrient for pathogenic bacteria and is not readily available within the human host because it is located intracellularly and sequestered by a variety of iron-bind- host proteins, such as hemoglobin and transferrins. One of the means by which S. pyogenes has been proposed to obtain essential iron for growth is via SiaA, also referred to as HtsA, which is part of an ABC transporter involved in iron acquisition that acts by binding host hemoproteins. Liu and Lei (23) proposed that Shp, a streptococcal cell surface protein which is encoded on the same operon as siaA, acquires heme by binding hemoglobin and subsequently transferring the heme to SiaA (23).

Streptolysin S (SLS) production has also been proposed as an important part of the mechanism used by S. pyogenes to acquire intracellular iron by lysing host red blood cells. SLS, an oxygen-stable, nonimmunogenic hemolysin with a broad cytolytic spectrum, is encoded by a nine-gene sag operon that was shown to be essential and sufficient for the production of SLS. In addition to its putative role in iron acquisition, SLS is thought to be important for the pathogenesis of S. pyogenes because it is involved in inflammation, tissue injury, and resistance to phagocytic killing.

The sagA gene, also referred to as pel, encodes a bacteriocin-like peptide which functions as the basic structural unit of SLS. Further, sagA mRNA has also been implicated as a regulatory molecule that propose to have an effect on virulence factors, such as M proteins, Sig, and SpeB. Since sagA was proposed as a regulatory, bacteriocin-like molecule and we have previously shown that its expression increases with increasing cell density, it could act as a quorum-sensing molecule.

Quorum sensing is a density-dependent process which involves chemical signaling molecules that reach a critical threshold concentration with increasing cell density, resulting in altered gene expression. In gram-positive bacteria, signal molecules are usually oligopeptides secreted extracellularly by an ABC transporter. The peptide is detected by a two-component signal transduction system consisting of a sensor kinase and a response regulator, which relay the signal intracellularly, whereby the sensor kinase phosphorylates the response regulator, thus activating it. The phosphorylated response regulator then binds to DNA and alters target gene expression. Interestingly, several bacteriocins, which are a class of antimicrobial peptides produced by bacteria, including nisin of Lactococcus lactis and subtilin of Bacillus subtilis, were shown to be regulated by quorum sensing. In fact, the structural peptides of nisin and subtilin were shown to function as the
signaling molecules that induced their own expression upon activation of the density-dependent autoinduction loop (18). We propose that the bacteriocin-like \textit{sagA} functions in a similar manner, whereby the structural peptide SLS induces its own expression in a density-dependent manner.

This study was undertaken to explore how \textit{S. pyogenes} regulates the expression of \textit{sagA} and \textit{siaA} in response to various iron concentrations and cell densities. Furthermore, we hypothesized that \textit{sagA} encodes a quorum-sensing signaling peptide based on its deduced peptide features, predicted cleavage, and posttranslational modifications (27) and on its response to cell density (3). We found that \textit{siaA} was up-regulated in response to limiting iron conditions, thus adding strength to its role as a gene involved in iron acquisition. Moreover, \textit{sagA} was shown to respond as a quorum-sensing signaling peptide since it was up-regulated both in a density-dependent manner and by SLS and was not induced in conditioned media from \textit{sagA}-deficient mutants.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** \textit{S. pyogenes} MGAS166 (M1 serotype) was used as the test strain for all in vitro experiments discussed in this study. Two strains, SBHN5(\textit{ALSLS}) (a nonhemolytic derivative of MGAS166 with a Tn916 insertion in the promoter region of the \textit{sga} operon) (3), and NZ131:sagA::cat (an M49-serotype group A Streptococcus with an in-frame algal replacement of \textit{sagA} by chloramphenicol) (7), were utilized to generate conditioned media. For all in vitro analyses of gene expression, overnight cultures of MGAS166 were grown in Todd-Hewitt (TH) broth (Difco Laboratories, MI) centrifuged at 3,910 \( \times \) g at 4°C for 10 min, washed twice with either fresh TH broth or iron-restricted TH broth, and resuspended in the same. This overnight suspension was used at a dilution of 1/20 to inoculate the test media, incubated at 37°C in ambient air, and sampled for mRNA at various time points (2, 4, and 6 h postinoculation).

**Preparation of test medium conditions.** In this study, TH broth was considered nonconditioned medium. Conditioned medium was prepared by growing MGAS166, SBHN5(\textit{ALSLS}), or NZ131:sagA::cat in TH broth at 37°C in ambient air for 6 h (optical density at 600 nm \([OD_{600}]\) of 0.6 to 0.8). This culture was then centrifuged at 3,910 \( \times \) g at 4°C for 15 min, and the cell-free supernatant was removed and filter sterilized using a Stericup filtration system (Millipore, Ontario, Canada) in accordance with the recommendations of the supplier. Controls for cDNA synthesis included a no-RNA template sample and one without reverse transcriptase. The real-time PCR assays were performed in triplicate on each of the duplicate samples by using a SmartCycler system (Cepheid, Sunnyvale, CA) and a Quantitect SYBR green PCR kit (QIAGEN, Ontario, Canada). Each 25-\( \mu \)l reaction mixture included 2 \( \mu \)l of cDNA (200 ng), 250 nmol of each primer (Table 1), and 2 \( \times \) SYBR green mix. The reactions were cycled in the Smart-Cycler by using the following parameters: 95°C for 15 min for the hot start, followed by 40 cycles of 94°C for 30 s, annealing at optimal temperature (Table 1) for 30 s, and primer extension at 72°C for 30 s. Gene expression analysis included the generation of standard curves for each gene and the utilization of the DNA gyrase A gene (\textit{gyrA}) as an internal standard for normalizing gene expression, as described previously (30).

**Statistical analysis.** Statistical analysis was conducted using a single factor analysis of variance (ANOVA) such that relative expression levels were compared against 1.0, which indicates no change or equal levels of expression between experimental conditions being analyzed.

**RESULTS**

**Growth kinetics of MGAS166.** The conditions used to examine gene expression included nonconditioned medium and conditioned medium from \textit{sagA} wild-type and mutant strains, varying iron concentrations, and the addition of SLS. Thus, to ensure that the growth conditions selected for gene expression analysis did not affect the growth of MGAS166, the growth kinetics of this organism were analyzed. MGAS166 was able to grow in the conditioned medium from MGAS166, SBHN5(\textit{ALSLS}), and NZ131:sagA::cat with increasing cell densities from 0 to 6 h postinoculation (Fig. 1A). Similarly, since various concentrations of SLS, ranging from 0.1 to 5.0 \( \mu \)g/ml, did not inhibit the growth of MGAS166 (Fig. 1B), gene expression was analyzed in 5.0 \( \mu \)g/ml of SLS. A range of iron concentrations from 1.0 \( \mu \)M to 1,000 \( \mu \)M did not appear to alter the growth kinetics of MGAS166 (Fig. 1C). Since iron is necessary for growth and medium supplemented with essential divalent cations (such as CaCl\textsubscript{2}, MgCl\textsubscript{2}, MnCl\textsubscript{2}, and ZnCl\textsubscript{2}) results in the presence of trace iron, we chose to test gene expression at 1.0 and 1,000 \( \mu \)M of iron. Thus, relative expression of \textit{sagA} and \textit{siaA} was measured in low (1.0 \( \mu \)M) and high (1,000 \( \mu \)M) concentrations of iron.

**Gene expression in nonconditioned medium.** Both \textit{sagA} and \textit{siaA} gene expression in MGAS166 were analyzed in vitro for up to 6 h in nonconditioned medium. With increasing cell

<table>
<thead>
<tr>
<th>Gene</th>
<th>Optimal annealing temp (°C)</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>sagA</td>
<td>50</td>
<td>5'-AGGAGGTAAACCTTATGTA-3'</td>
<td>5'-TACCACTTGGAAATTCACA-3'</td>
</tr>
<tr>
<td>siaA</td>
<td>65</td>
<td>5'-CAGCAGAGATTTGTCACCTGCG-3'</td>
<td>5'-CACAACGCTTAACAGCATCATAG-3'</td>
</tr>
<tr>
<td>gyrA</td>
<td>65</td>
<td>5'-AGCAGACAGATGTCATTGTGCTAG-3'</td>
<td>5'-CCAGTCAAACGCGAAACG-3'</td>
</tr>
</tbody>
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**TABLE 1. Primers and optimal annealing temperature used for the real-time PCR analysis of \textit{sagA}, \textit{siaA}, and \textit{gyrA}**
densities, an increase in the expression of both genes was observed (Fig. 2). The gyrA-normalized expression of siaA increased approximately 10-fold from 0.006 at 2 h to 0.070 at 6 h postinoculation (Fig. 2A). Similarly, the normalized sagA expression increased roughly fivefold from 0.044 at 2 h to 0.21 at 6 h postinoculation (Fig. 2B).

Gene expression in conditioned medium. To determine whether sagA regulates gene expression in a density-dependent manner, gene expression was analyzed in the MGAS166 wild-type strain grown in conditioned medium from MGAS166 wild-type and from two sagA-deficient mutants (SBNH5[ΔSLS] and NZ131:sagAΔcat). The relative levels of sagA and siaA gene

FIG. 1. Growth kinetics of MGAS166 in various conditioned media (A) and various concentrations of SLS (B) and ferric chloride (C). Each experiment represents an average of three independent cultures. Error bars indicate standard deviations.

FIG. 2. Average normalized expression of siaA (A) and sagA (B) in MGAS166 from duplicate cultures grown in nonconditioned medium at 2, 4, and 6 h postinoculation. Both siaA and sagA expression levels were normalized with gyrA. Error bars indicate standard deviations.
expression were determined by a comparison of normalized gene expression in conditioned medium relative to that in nonconditioned medium. \textit{sagA} was expressed at significantly higher levels in conditioned medium from MGAS166 relative to growth in nonconditioned medium throughout the time period tested (Fig. 3A). This dramatic up-regulation of \textit{sagA} ranged from 11.3-fold at 2 h postinoculation to 22.4-fold at 6 h postinoculation. This relative up-regulation of \textit{sagA} was not observed when MGAS166 was grown in the conditioned medium from the \textit{sagA} mutants (Fig. 3B and C). In fact, there was no significant difference between the levels of \textit{sagA} gene expression in these conditioned media relative to that in nonconditioned medium. By contrast, \textit{siaA} expression was up-regulated in the conditioned media from MGAS166 as well as in media from the \textit{sagA}-deficient mutants (Fig. 3). Interestingly, this up-regulation of \textit{siaA} occurs earlier during the growth phase at 2 and 4 h postinoculation. Furthermore, there was no significant difference in the level of \textit{siaA} expression at 6 h postinoculation in each of the conditioned media (MGAS166, SBNH5[\Delta SLS], and NZ131:\textit{sagA}\Delta\textit{cat}) relative to that in the nonconditioned medium.

**Gene expression in 5.0 \(\mu\text{g/ml}\) of SLS.** The expression of \textit{sagA} and \textit{siaA} was determined in MGAS166 grown in the presence of 5.0 \(\mu\text{g/ml}\) SLS, the gene product of \textit{sagA}, to confirm its role as a signaling molecule. SLS up-regulated the expression of \textit{sagA} at 2, 4, and 6 h postinoculation relative to its expression in nonconditioned medium (Fig. 4B). Although \textit{siaA} was also up-regulated by SLS at 2, 4, and 6 h postinoculation, the highest level of \textit{siaA} relative expression occurred at 2 h postinoculation and the lowest occurred at 6 h postinoculation, which was in contrast to \textit{sagA} (Fig. 4A).

**Gene expression in iron-restricted medium.** The effect of iron (ferric chloride and ferric citrate) on gene expression was analyzed in medium containing low (1.0 \(\mu\text{M}\)) iron concentration relative to medium containing high (1,000 \(\mu\text{M}\)) iron concentration. The \textit{siaA} gene was significantly up-regulated in the presence of lower (1.0 \(\mu\text{M}\)) relative to higher (1,000 \(\mu\text{M}\)) concentrations of ferric chloride and ferric citrate at 2 and 4 h postinoculation, respectively (Fig. 5A). By 6 h postinoculation, there was no significant difference in the level of \textit{siaA} expression in the presence of 1.0-\(\mu\text{M}\) and 1,000-\(\mu\text{M}\) concentrations of iron. Conversely, \textit{sagA} was significantly down-regulated at 1.0 \(\mu\text{M}\) of ferric chloride relative to at 1,000 \(\mu\text{M}\) of ferric chloride (Fig. 5B). The down-regulation of \textit{sagA} by low ferric citrate concentration relative to high ferric citrate concentration was evident at 4 h postinoculation (Fig. 5B).

**DISCUSSION**

We explored the influence of iron and cell density on the expression of two virulence factors, \textit{sagA} and \textit{siaA}. The initial analysis of \textit{sagA} and \textit{siaA} expression in nonconditioned culture medium from 2 to 6 h indicated that the expression of each gene increased in a growth phase-dependent manner (Fig. 2),
which is characteristic of quorum-sensing signaling molecules. However, when MGAS166 was grown in conditioned medium from the same strain, *sagA* but not *siaA* was expressed at significantly higher levels relative to growth in nonconditioned medium throughout the time period tested (Fig. 3A). Furthermore, *sagA* expression in conditioned medium also increased with increasing cell density from 2 to 6 h postinoculation. We confirmed that the cell density of the culture during growth was not inhibited by the conditioned medium (Fig. 1A). These results are consistent with the hypothesis that *sagA* encodes or influences activity of a signal molecule present in the conditioned medium and that the increased expression noted during laboratory growth is a result of increased cell density. In contrast, *siaA* was up-regulated only at 2 h postinoculation in the conditioned medium (Fig. 3A). Furthermore, by 4 and 6 h postinoculation, *siaA* expression returned to levels similar to that in nonconditioned medium. This pattern of up-regulation is not consistent with that of a quorum-sensing signaling molecule, which increases in expression in a growth-dependent manner.

The differences in gene expression between conditioned and nonconditioned medium suggested that the conditioned medium contained a signaling molecule; however, we did not conclusively identify the *sagA* gene product as being this molecule. In order to demonstrate *sagA* as a signaling molecule, MGAS166 was grown in conditioned media from *sagA*-deficient mutants of two serotypes (M1 and M49). These mutants differed slightly in their genetic backgrounds as the M1 mutant contained a transposon insertion in the *sag* operon promoter region that resulted in a mutation, which abrogated expression of the entire nine-gene operon (3). The M49 strain had a nonpolar in-frame deletion of the *sagA* gene, with the eight downstream genes being expressed (7). Most importantly both strains failed to produce an active SLS. We selected mutants of two different serotypes to determine whether varying the M serotype would alter the affect on *sagA* expression, particularly since the regulatory effect of *sagA* might be serotype dependent (3, 4, 7, 9, 21). The conditioned media from the mutants were characterized by the absence of a *sagA* transcript or SLS that is the product of this operon. In the absence of SLS, the conditioned medium did not up-regulate *sagA* expression relative to nonconditioned medium (Fig. 3B and C), indicating that it is indeed the *sagA* operon that is responsible for up-regulation. In contrast, there was little difference in the relative expression of *siaA*, regardless of whether the conditioned me-
dium was from the MGAS166 parent strain or the sagA-deficient mutants (Fig. 3).

Our data differs from those of Mangold et al. (24) who found that the addition of conditioned medium from a sagA-deficient mutant to an M1 serotype wild-type strain resulted in the up-regulation of sagA (24). However, by adding conditioned medium in a ratio of 1:1 to lag-phase cultures, these authors did not eliminate any inducing signals from the lag-phase cultures. We found that the addition of lag-phase cultures to conditioned medium also resulted in the up-regulation of sagA (data not shown). Furthermore, by using the conditioned medium immediately after preparation, we eliminated any potential effects that could result from freezing. Finally, real-time PCR analysis of mRNA expression is more sensitive than Northern analysis and avoids the problem of equalizing total cell numbers since the gyrA gene allows for the standardization of mRNA expression levels. Thus, we attribute this inconsistency between our results and those of Mangold et al. (24) to likely result from differences in experimental procedures.

Since SLS is the functional product of the sagA operon, its effect on gene expression was also evaluated. Various concentrations of exogenously added SLS were initially tested to ensure that the addition of SLS did not alter the cell density of the culture medium (Fig. 1B). The expression of sagA increased after the addition of SLS relative to nonconditioned medium, and this up-regulation followed a growth phase-dependent increase from approximately 1.5-fold at 2 h to roughly 5-fold at 6 h (Fig. 4B). Despite the presence of impurities in commercial SLS due to difficulties in purifying this toxin (8), its exogenous addition caused the induction of sagA similar to the pattern of induction observed with conditioned medium from the MGAS166 wild type. Interestingly, at 5.0 μg/ml, SLS did not induce sagA to levels as high as those in MGAS166-conditioned medium. The reasons for this result could be that (i) the concentration of SLS used for this analysis was either too high or too low or (ii) the presence of impurities in SLS could have altered its activity. Nevertheless, our data provide convincing evidence that the sag operon is indeed involved in signaling.

The addition of commercially available SLS also dramatically up-regulated siaA, and its effect was not growth phase dependent because the highest level of up-regulation (approximately 130-fold) was observed at 2 h rather than at 6 h (approximately 10-fold) (Fig. 4A). This result suggests that siaA could be a target gene for the SLS quorum-sensing system. Furthermore, the fact that siaA was not up-regulated in a growth phase-dependent manner indicated that it was not responding as a signaling molecule.

In addition to cell density and SLS, another environmental stimulus investigated in this study was iron, which has been studied poorly in relation to the virulence of S. pyogenes. Interestingly, one of the earliest associations between hemolysin production and iron acquisition by S. pyogenes was made by Griffiths and McClain (14). They utilized dilaoyzed brain heart infusion, which was chelated of all ions and then supplemented with essential cations and iron concentrations of up to 5.0 μg/ml, to determine the effect of iron concentration on hemolysin production. Griffiths and McClain showed that the hemolytic activity of S. pyogenes was affected by iron concentration, suggesting that the sag operon responded to iron as an extra-cellular signal. Furthermore, the hemolytic ability of S. pyogenes has been proposed as a means for this organism to acquire iron by lysing host cells (2, 10).

Iron additions at 1.0 and 1,000 μM were selected for relative gene expression analysis since neither appeared to significantly affect the growth kinetics of MGAS166 (Fig. 1C). Relative to higher iron concentrations, lower iron concentrations induced the expression of siaA but not of sagA (Fig. 5). These data are consistent with the finding that siaA is induced under limited iron conditions (2, 19). The sagA gene, however, was not up-regulated under the lower iron concentrations, though it was up-regulated at higher iron concentrations. There are two possible explanations for this result. First, once iron becomes accessible (low-iron concentrations) through uptake by the sia operon or another iron acquisition system of S. pyogenes, SLS need not be up-regulated to lyse red blood or other host cells in order to release intracellular iron. Second, one of the circumstances during which S. pyogenes could encounter conditions of high-iron concentrations during an infection is within macrophages following phagocytosis. Once phagocytosed, a bacterium is exposed to high levels of iron within the macrophages; this exposure stimulates the formation of damaging reactive oxygen species through the Fenton reaction (28). Although S. pyogenes possesses defense mechanisms against oxidative stress (12, 13, 16, 17, 29), it would also be advantageous for the organism to increase the production of its potent cytolytic SLS to allow it to escape from the macrophages. S. pyogenes has been shown to escape from the phagocytic vacuoles of polymorphonuclear leukocytes and escape into the cytoplasm where the bacteria not only remain viable but also are able to multiply (25). Although the mechanism by which this occurs is not known, it is possible that SLS plays a role in the process.

In conclusion, we demonstrated that sagA responds in a quorum-sensing manner, whereas siaA is stimulated under reduced iron conditions. Although SLS induced the aforementioned genes, only sagA expression responded in a growth-dependent manner. Future work analyzing the interplay between hemolysin production and iron acquisition will be useful for providing a comprehensive understanding of this pathogen.

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