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Inactivation of the group A *Streptococcus* regulator *srv* results in chromosome wide reduction of transcript levels, and changes in extracellular levels of Sic and SpeB

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Abstract

Group A *Streptococcus* is characterized by the ability to cause a diverse number of human infections including pharyngitis, necrotizing fasciitis, toxic shock syndrome, and acute rheumatic fever, yet the regulation of streptococcal genes involved in disease processes and survival in the host is not completely understood. Genome scale analysis has revealed a complex regulatory network including 13 two-component regulatory systems and more than 100 additional putative regulators, the majority of which remain uncharacterized. Among these is the streptococcal regulator of virulence, *Srv*, the first Group A *Streptococcus* member of the Crp/Fnr family of transcriptional regulators. Previous work demonstrated that the loss of *srv* resulted in a significant decrease in Group A *Streptococcus* virulence. To begin to define the gene products influenced by *Srv*, we combined microarray and two-dimensional gel electrophoresis analysis. Loss of *srv* results in a chromosome wide reduction of gene transcription and changes in the production of the extracellular virulence factors Sic (streptococcal inhibitor of complement) and SpeB (cysteine proteinase). Sic levels are reduced in the *srv* mutant, whereas the extracellular concentration and activity of SpeB is increased. These data link *Srv* to the increasingly complex GAS regulatory network.

Keywords

group A *Streptococcus*; virulence gene regulation; streptococcal regulator of virulence, *Srv*; *Streptococcus pyogenes*

Introduction

Group A *Streptococcus* (GAS) is perhaps best recognized as a leading cause of pediatric pharyngitis when, in fact, the organism may cause a greater number of human diseases than any other bacterial pathogen (Ferretti *et al.*, 2001). In addition to pharyngitis, these infections can range from those associated with the skin (impetigo, erysipelas), to severe infections such as necrotizing fasciitis and streptococcal toxic shock syndrome. This diverse epidemiology speaks to the ability of GAS to colonize numerous physiologically distinct host tissue sites. Both population genetic and genomic studies have attempted to identify specific genetic markers that would define segments of the GAS population as those responsible for mild infections of the skin vs. those frequently involved in invasive episodes (Bessen *et al.*, 1989, 1996, 1999, 2000; Ferretti *et al.*, 2001; Beres *et al.*, 2002; Smoot *et al.*, 2002; Nakagawa *et al.*, 2003; Banks *et al.*, 2004; McGregor *et al.*, 2004). However, no simplistic association with a given clinical syndrome has been made, and genomic studies have failed to identify specific virulence factors that explain certain GAS strain/disease associations (Ferretti *et al.*, 2001; Beres *et al.*, 2002; Smoot *et al.*, 2002; Nakagawa *et al.*, 2003; Banks *et al.*, 2004; McGregor *et al.*, 2004). Genome sequencing has revealed an intricate gene regulatory network including 13 putative two-component systems and up to 100 other regulatory proteins (Graham *et al.*, 2002). This observation leads to the hypothesis that strain-to-strain differences in gene regulation may be responsible for the frequency with which certain strains or serotypes are associated with disease. Of course, before such a hypothesis can be addressed, a systematic study of the regulatory elements of GAS must be undertaken.

Recently, we identified the first Crp/Fnr-like regulator of GAS named the streptococcal regulator of virulence (Srv) based upon work that indicated Srv was required for GAS virulence in a mouse model of infection (Reid *et al.*, 2004). *srv* (*spy1857*) was originally identified in a sequence analysis of completed GAS genomes (Reid *et al.*, 2001a, b). The gene is most similar to *prfA*, the primary regulator of virulence in *Listeria monocytogenes* (Reid *et al.*, 2004). An analysis of 37 genetically diverse GAS strains indicated that *srv* encoded only two protein variants differing by a single amino acid, a result that suggests strong selective pressure to maintain Srv function (Reid *et al.*, 2001a, b). Crp/Fnr-like regulators are site-specific DNA-binding proteins characterized by an N-terminal allosteric domain and a C-terminal helix-turn-helix domain required for interaction with DNA. Sequence analysis indicates *srv* encodes an N-terminal region consisting of short β -sheets separated by conserved glycine residues (β -roll structures), and a C-terminal helix-turn-helix domain (Reid *et al.*, 2004).

Numerous bacterial regulators have been identified through studies designed to determine the underlying cause of a specific phenotype. For instance, *prfA* was identified through the study of a 450 bp deletion in a nonhemolytic strain of *L. monocytogenes* (Leimeister-Wachter *et al.*, 1990). This deletion was located 1.6 kb upstream of the listeriolysin gene and found to interrupt a coding region (*prfA*) required for listeriolysin expression (Leimeister-Wachter *et al.*, 1990). Similarly, *mga*, a virulence regulator of GAS, was identified through the study of strains which failed to produce M protein (Spanier *et al.*, 1984; Caparon & Scott, 1987; Robbins *et al.*, 1987; Simpson & Cleary, 1987). However, *srv* was identified through genome scanning. Allelic replacement of *srv* resulted in an attenuated mutant strain in an intraperitoneal model of GAS infection, but it was unclear which Srv-dependent genes were responsible for the significant reduction in virulence (Reid *et al.*, 2004). To address this issue, we conducted a microarray analysis comparing global gene transcript levels in a serotype M1 strain (MGAS5005) and an isogenic mutant derivative lacking *srv* (MGAS5005 Δ *srv*). In cultures harvested during the exponential phase of growth ($A_{600\text{ nm}} = 0.4$), 308 genes were downregulated in the *srv* mutant strain whereas four genes were upregulated. Among the downregulated genes were several which encode proven/putative extracellular proteins. To analyze the role of Srv in the regulation of GAS extracellular proteins, a two-dimensional gel

electrophoresis study was completed. Taken together, the analyses suggest that Srv is a regulator of GAS gene transcription and impacts the GAS extracellular protein profile, results which may account for the reduction in virulence of the *srv* mutant strain.

Materials and methods

Bacterial strains and growth conditions

Serotype M1 strain MGAS5005 and the MGAS5005 Δ *srv* derivative have been described (Reid *et al.*, 2004). Of particular note, population genetic analysis and epidemiological studies have indicated that serotype M1T1 strains are among the most common causes of invasive GAS infections worldwide. MGAS5005 was isolated from a case of invasive GAS disease and has been extensively characterized in numerous investigations of GAS pathogenesis (Musser & Krause, 1998; Reid *et al.*, 2001a, b).

For RNA isolation, GAS was cultured in Todd–Hewitt broth supplemented with yeast extract (THY) (Difco Laboratories, Detroit, MI) overnight at 37 °C, 5% CO₂. A 100- μ L aliquot of the starter culture was added to each of three 50-mL aliquots of THY medium, incubated at 37 °C, 5% CO₂, and harvested at three time points ($A_{600\text{ nm}}$ = 0.2, 0.4, 0.6) throughout the growth cycle. Cells were harvested by centrifugation and frozen at –80 °C prior to RNA isolation.

For protein isolation, MGAS5005 and MGAS5005 Δ *srv* were grown with 40 mL THY broth for *c.* 18 h at 37 °C, 5% CO₂. The cultures were centrifuged for 15 min at 13 679 *g* at 4 °C to pellet the bacteria. The culture supernatant fluids were transferred to a sterile centrifuge tube and ice-cold trichloroacetic acid and acetone were added to final concentrations of 10% and 5% (v/v), respectively. The samples were mixed and incubated for 1 h at –20 °C prior to centrifugation for 20 min at 1500 *g* and 4 °C. The pellets were washed twice with ice-cold ethanol and resuspended with solubilization solution containing 8 M urea, 2 μ thiourea, 4% CHAPS, and 75 μ M dithiothreitol (DTT). Proteins were quantitated with a PlusOne 2-D Quant kit (Amersham Biosciences, Piscataway, NJ), as described by the manufacturer.

Microarray analysis

The microarray consisted of 2215 PCR products ranging from 100 to 500 bp amplified from MGAS5005 ORFs based on strain SF370 (Ferretti *et al.*, 2001), and unique M18 (MGAS8232) and M3 (MGAS315) ORFs (Beres *et al.*, 2002; Smoot *et al.*, 2002). PCR products derived from each GAS ORF were printed onto glass slides (CMT-GAPS, Corning) using a VersaArray Chipwriter Pro (Bio-Rad Laboratories, Hercules, CA) in $\geq$ quadruplicate. First-strand cDNA synthesis (incorporation of biotin and fluorescein probes) and tyramide signal amplification (TSATM) was performed using MicromaxTM TSATM Labeling and Detection Kit (Perkin Elmer Life Sciences, Boston, MA). Hybridization was conducted overnight in a 45 °C water bath. Lifterslips (Erie Scientific, Portsmouth, NH) were removed by immersion in a solution of 0.5 $\times$ sodium sodium citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS) at 45 °C, washed once in the same solution, followed by additional washes in 0.06% $\times$ SSC/0.01% SDS, and 0.06 $\times$ SSC followed by amplification of label using TSATM detection. Slides were scanned with a ScanArray 5000 instrument (Perkin Elmer Life Sciences) and fluorochromes were normalized to equal fluorescence intensity using serial dilutions of genomic DNA standards by adjusting laser power and/or photomultiplier gain. Spot location and statistics were calculated and exported from QuantArray (Perkin Elmer Life Sciences), then imported into GENESPRING version 6 (Silicon Genetics, Redwood City, CA). Mean values from replicates were calculated and all genes identified as differentially expressed passed a custom filter based on spot intensity, signal to noise ratio, and area (Voyich *et al.*, 2003). The experiments were performed in triplicate with each slide containing four to 12 spots per gene ($n \geq 12$ spots).

TaqMan real-time reverse transcriptase-PCR

Total RNA used in the microarray analysis was also used for verification of the array by real-time reverse transcriptase-PCR. TaqMan assays were performed with an ABI 7700 instrument (Applied Biosystems, Forest City, CA) and the TaqMan One-Step RT-PCR master mix reagents kit (Applied Biosystems) as previously described (Reid *et al.*, 2001a, b, 2004). The amplification profile used was as follows: 1 cycle at 48 °C for 30 min, 1 cycle at 95 °C for 10 min, and 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The critical threshold cycle (C_t) is defined as the cycle at which fluorescence becomes detectable above background and is inversely proportional to the logarithm of the initial concentration of template. A standard curve was plotted for each reaction with C_t values obtained from amplification of known quantities of genomic DNA isolated from strain MGAS5005. The standard curves were used to transform C_t values of the experimental samples into the relative number of DNA molecules. The quantity of cDNA for each experimental gene was normalized to the quantity of the constitutively transcribed control gene (*gyrA*) in each sample. The fold change in transcript levels was determined by taking the ratio of the level of cDNA in MGAS5005 Δ *srv* to the level of cDNA in MGAS5005.

Two-dimensional electrophoresis

Isoelectric focusing was done with an IPGphor isoelectric focusing system using immobilized dry strips (11 cm) with a linear pH range of 3–10, as described by the manufacturer (Amersham Biosciences). IPG strips were hydrated with 90 μ g of protein in 200 μ L of sample buffer for 12 h at 20 °C. Isoelectric focusing was done with 500 V for 500 Vh, 1000 V for 1000 Vh, and 8000 V for 24 000 Vh at 20 °C. The strips were incubated in SDS equilibration buffer (50 mM Tris-Cl [pH 8.8], 6 μ M urea, 30% (v/v) glycerol, 2% SDS, and Bromophenol blue) for 10 min. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) separation was done with a Criterion Dodeca electrophoresis apparatus (Bio-Rad Laboratories) and 12.5% Criterion precast acrylamide resolving gels (Bio-Rad Laboratories). The running buffer consisted of 0.25 μ M Tris, 1.92 μ M glycine, and 1% (w/v) SDS and electrophoresis was done for *c.* 2 h at 100 V. Proteins were stained with Sypro Ruby (Bio-Rad Laboratories), and digital images were acquired with a Typhoon imager (Amersham Biosciences). Analysis of the gels, including protein spot detection and quantitation, was done with PDQUEST software (Bio-Rad Laboratories). Protein spots were quantitated by summing the values of pixels of each protein spot. Gels were normalized based on the sum of all protein spots detected in each sample. The experiment was repeated on three separate occasions.

Protein identification

Proteins of interest were excised from the SDS-PAGE gels, digested with trypsin, and identified, as previously described (Chaussee *et al.*, 2004). Briefly, tryptic peptides were separated with a C18 reverse-phase column (13 $\times$ 25 μ m; LC Packings, Sunnyvale, CA) using a capillary liquid chromatography system (Waters, Milford, MA) and eluted directly into a Micromass electrospray ionization quadrupole/orthogonal time-of-flight mass (Q-ToF Micro) hybrid spectrometer (Waters) with a 3–40% gradient of ACN-0.1% formic acid over 40 min and a flow rate of *c.* 20 nL min⁻¹. Spectra were obtained in positive ion mode, deconvoluted, and analyzed with MASSLYNX 4.0 software (Micromass). Protein Lynx Global Server v. 2.1 (Waters) was used to search the NCBI nonredundant genomic databases. Proteins were identified by matching mass spectrometry (MS)/MS spectra from at least two tryptic peptides or by *de novo* peptide sequence determination when only one MS/MS match was identified.

Casein agar plate assay

The proteolytic activity of SpeB was determined as described by Hynes & Tagg (1985). Briefly, MGAS5005 and MGAS5005 Δ *srv* were stab inoculated into Todd–Hewitt agar plates

containing 10% skim milk (Difco Laboratories), and incubated anaerobically at 37 °C for c. 18 h in an anaerobic GasPak (Becton Dickinson) (Hynes & Tagg, 1985). A zone of translucence around the stab site indicated the presence of caseinolytic activity.

Western immunoblot analysis of SpeB in culture supernatant fluids

MGAS5005 and MGAS5005 Δ *srv* were grown in 40 mL of THY broth at 37 °C with 5% CO₂. Culture supernatant proteins were precipitated and prepared as described above for 2-DE. A 20- μ g aliquot of protein was separated with SDS-PAGE and the proteins transferred to nitrocellulose membranes (Millipore Corp., Bedford, MA) with Towbin's buffer. Rabbit antisera to SpeB was used at a 1 : 2000 dilution. The antisera was raised against the zymogen form of SpeB and reacts with both the active and zymogen forms. Antibody-antigen complexes were detected with Super-Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL) and images acquired with a Typhoon imager (Amersham Biosciences).

Results

Inactivation of *srv* alters the GAS transcription profile

As a first step in defining the spectrum of genes that may be influenced by Srv, we conducted a microarray analysis of GAS gene transcript levels in MGAS5005 and MGAS5005 Δ *srv*. In previous work, we demonstrated that the maximal level of *srv* gene transcript was detected at two distinct times, early- and late-exponential phase ($A_{600\text{ nm}} = 0.1$ and $A_{600\text{ nm}} = 0.6$, respectively) (Reid *et al.*, 2004). Therefore, we chose to harvest GAS at $A_{600\text{ nm}} = 0.2, 0.4$, and 0.6 , time points, which coincide with among the highest ($A_{600\text{ nm}} = 0.6$) and lowest ($A_{600\text{ nm}} = 0.2$ & 0.4) measured levels of *srv* transcript (the quantities of RNA that could be isolated from a culture harvested at $A_{600\text{ nm}} = 0.1$ were insufficient to permit microarray analysis). To control for variation in individual RNA isolations, total RNA was isolated from triplicate cultures at each time point. Hybridizations were done in duplicate for each of the triplicate RNA samples at each time point. Spot signal intensities minus local background fluorescence were normalized to the global signal intensity per label before data analysis. Transcript expression ratios (or fold change) were calculated for the *srv* mutant strain relative to MGAS5005. The procedures used to conduct this study have been published previously in other reports of GAS microarray analysis (Smoot *et al.*, 2001, 2002; Graham *et al.*, 2002).

A recent report indicates that less than a 2-fold change in relative transcript abundance can be biologically significant (Hughes *et al.*, 2000). In addition, a previous GAS micro-array analysis utilizing a 1.5-fold threshold revealed that growth temperature influenced the expression of genes involved in iron homeostasis (Smoot *et al.*, 2001). Given that our study was designed to assess the regulatory potential of Srv, we elected to use a 1.5-fold threshold (Table S1). Chromosome wide, only 24 genes were differentially transcribed at $A_{600\text{ nm}} = 0.2$ (18 downregulated, 6 upregulated). However, a dramatic change in the GAS transcriptome was observed at $A_{600\text{ nm}} = 0.4$ with 18% of genes (308/1678) downregulated in the *srv* mutant; 54 of those genes were downregulated ≥ 2 -fold. Furthermore, only four genes were upregulated in the mutant strain at $A_{600\text{ nm}} = 0.4$ (Table 1). A similar trend was observed at $A_{600\text{ nm}} = 0.6$: 13% of GAS genes were downregulated in the *srv* mutant (224/1678), 43 genes were downregulated ≥ 2 -fold, and only a single ORF was upregulated.

To gain further understanding of the nature of the changes that were taking place in MGAS5005 Δ *srv*, genes which were downregulated in the mutant strain were classified according to the COG (clusters of orthologous groups) database (Table 2). This system predicts function based upon phylogenetic relationships and assigns the genes to broad categories of basic cellular processes (Tatusov *et al.*, 2000). According to this analysis, the categories with the greatest percentage of genes downregulated were translation; posttranslational

modification, protein turnover, and chaperones; energy production and conversion; amino acid transport and metabolism; lipid transport and metabolism; and secondary metabolites biosynthesis, transport and catabolism (Table 2). In addition, 18 putative or proven virulence factors (Table 3) were found to be downregulated at various time points in MGAS5005 Δ *srv*.

Verification of DNA microarray

Real-time reverse transcriptase-PCR analysis of five genes was used to confirm the microarray data. Total RNA was isolated from MGAS5005 and MGAS5005 Δ *srv* at $A_{600\text{ nm}} = 0.4$ and 0.6 to correspond with the time points generating the greatest differences in gene transcript observed in the array analysis. Two genes were analyzed at $A_{600\text{ nm}} = 0.4$, and three separate genes were analyzed at $A_{600\text{ nm}} = 0.6$. At $A_{600\text{ nm}} = 0.4$, the ratio of transcript levels (MGAS5005 Δ *srv* : MGAS5005) for SPy0338 was 0.504, and for SPy0714 was 0.611. The fold change in transcript for these two genes in the microarray analysis was 0.61 and 0.63, respectively. At $A_{600\text{ nm}} = 0.6$, the ratio of transcript levels (MGAS5005 Δ *srv* : MGAS5005) for SPy0044, SPy1869, and SPy2019 were 0.598, 0.578, and 0.344, respectively. In the microarray analysis, the fold change for these genes measured 0.64 (SPy0044), 0.70 (SPy1869), and 0.53 (SPy2019). Strong positive correlations between results obtained with the two techniques were observed ($r = 0.88$) (Fig. 1).

Inactivation of *srv* alters the profile of GAS proteins released to the supernatant

To test the hypothesis that inactivation of *srv* may result in changes in the number of products secreted by GAS, a Signal P analysis (<http://www.cbs.dtu.dk/services/SignalP/>) was used to identify genes, which encode a putative Gram-positive signal secretion sequence. Genes encoding 51 proven or putative secreted proteins of GAS were differentially regulated in MGAS5005 Δ *srv* according to the microarray. This observation led us to compare the exoprotein profiles of MGAS5005 and MGAS5005 Δ *srv*. Supernatant proteins were isolated from stationary phase cultures of MGAS5005 and MGAS5005 Δ *srv*. Stationary phase cultures were used because previous analyses of GAS culture supernatant fluids indicated that many soluble exoproteins are detected only in the stationary phase of growth (Chaussee *et al.*, 2001). The proteins were separated by SDS-PAGE (data not shown) and two-dimensional gel electrophoresis (2-DE). Slices of representative gels are shown in Fig. 2. The production of the serum inhibitor of complement (Sic) and the streptococcal cysteine proteinase (SpeB) consistently differed between MGAS5005 Δ *srv* and MGAS5005. Sic was less abundant among proteins isolated from MGAS5005 Δ *srv* than among those isolated from MGAS5005 (Fig. 2). These data are consistent with our microarray analysis, which indicated a fold change in transcript of 0.49 for *sic* (SPy2016) in the mutant strain compared to the wild type. Sic is encoded primarily by M1 serotypes, inhibits human complement activation *in vitro*, and contributes to virulence in murine models of disease (Akesson *et al.*, 1996; Stockbauer *et al.*, 1998; Hoe *et al.*, 2000, 2001, 2002; Lukomski *et al.*, 2000). In addition, SpeB was more abundant in samples prepared from MGAS5005 Δ *srv* cultures (Fig. 2). This result was unexpected in that there was no variation in the transcription of *speB* detected by either microarray or real-time reverse transcriptase-PCR (data not shown). The importance of SpeB in GAS pathogenesis was evidenced by the attenuation of the *speB* isogenic mutant (Lukomski *et al.*, 1997). SpeB has been shown to degrade host matrix proteins (Kapur *et al.*, 1993), and cleave antibacterial peptide LL-37 (Schmidtchen *et al.*, 2002), and IgG antibody (Collin *et al.*, 2002). Taken together, the data indicate that *srv* inactivation also is associated with changes in the abundance of virulence-associated exoproteins.

MGAS5005 Δ *srv* has greater SpeB caseinolytic activity

The observed increase in the production of SpeB in MGAS5005 Δ *srv* led us to investigate whether there was a correlated increase in SpeB activity. SpeB proteolytic activity was

measured by stab inoculating the strain to be tested into Todd–Hewitt agar plates containing 10% skim milk (casein agar plate assay) (Hynes & Tagg, 1985; Chaussee *et al.*, 1999). Greater caseinolytic activity, indicated by an increased zone of translucence surrounding the stab site, was observed in MGAS5005 Δ *srv* compared to the parental strain (Fig. 3). Thus, the increase in SpeB production in MGAS5005 Δ *srv* is accompanied by an increase in SpeB activity.

Western immunoblot analysis indicates that SpeB is produced earlier in MGAS5005 Δ *srv* than in the parental strain

The increase in caseinolytic activity is likely due to the increased concentration of SpeB present, but could indicate that SpeB is produced earlier in the MGAS5005 Δ *srv* background. To test this hypothesis, we conducted a western immunoblot analysis of GAS supernatants collected after 2 and 8 h of growth. After 2 h, SpeB was detected in supernatants isolated from the mutant strain, but not in supernatants obtained from the growth of MGAS5005 (Fig. 4). After 8 h, there is an increased level of SpeB present in the supernatant from MGAS5005 Δ *srv* compared to the wild-type strain (Fig. 4). This result suggests that Srv contributes to the temporal production of SpeB.

Discussion

Our analysis indicates that the absence of Srv results in a broad reduction in GAS gene transcript levels, with as much as 18% of genes downregulated in the mutant strain during the exponential phase of growth. Such large differences in expression in response to the inactivation of a single regulator are not unprecedented. Graham *et al.* (2002) reported that inactivation of *covR* (*csrR*) resulted in the differential expression of 15% of GAS genes ($n = 271$). In fact, 21 putative/proven transcriptional regulators were downregulated in MGAS5005 Δ *srv* during the exponential phase of growth, including *mga* and *covRS* (*csrRS*). The multiple gene regulator Mga has been associated with an array of GAS disease processes such as colonization, invasion, and evasion of the host immune response (Simpson & Cleary, 1987; Lukomski *et al.*, 2000; Smoot *et al.*, 2001; Collin *et al.*, 2002). Similarly, CovRS/CsrRS (control of virulence, capsule synthesis regulator) negatively regulates several factors that contribute to GAS pathogenesis such as SpeB, streptolysin S, and hyaluronic acid capsule production. Inactivation of *covRS* leads to a hypervirulent phenotype in murine models of GAS disease and resistance to complement-mediated killing by human polymorphonuclear leukocytes (Levin & Wessels, 1998; Heath *et al.*, 1999; Engleberg *et al.*, 2001; Graham *et al.*, 2002; Engleberg *et al.*, 2004). Why is there a reduction of virulence in the *srv* mutant strain (Reid *et al.*, 2004) when the level of *covRS/csrRS* transcripts is low? This is likely due to the combination genes that are downregulated in the *srv* mutant, including *mga*, effectively eliminating the hypervirulent phenotype associated with *covRS/csrRS* mutants (Engleberg *et al.*, 2001). Thus, while it is likely that a subset of the downregulated genes are Srv dependent, the overall reduction in transcript is a reflection of the reticulate regulatory network that exists in GAS (Graham *et al.*, 2002; Kreikemeyer *et al.*, 2003).

Our previous study of *srv* revealed that expression of the gene fluctuated with the highest levels of transcript identified at $A_{600\text{ nm}} = 0.1$ and $A_{600\text{ nm}} = 0.6$ (Reid *et al.*, 2004). However, we observed the greatest difference in gene transcription at $A_{600\text{ nm}} = 0.4$, a time when *srv* expression is at its lowest levels. There are several possible explanations for this observation:

1. A functional level of Srv is produced early in growth and then maintained *in vivo*. Levels of transcription increase in response to decreasing levels of Srv, suggesting that *srv* may be self-regulated and/or a sensory element may exist. Examination of the published SF370 (serotype M1), MGAS315 (M3), and MGAS8232 (M18) genome sequences does not reveal the presence of an adjacent ORF with homology to existing sensor kinases.

2. Srv is produced but remains inactive until interaction with an as yet unidentified cofactor. This cofactor may induce an oligomeric state required for function, or more subtle allosteric changes. These changes may allow Srv to interact with its own promoter.
3. Srv is regulated at the level of translation or undergoes posttranscriptional modification. Changes in *srv* transcription are in response to growth-phase, and do not reflect active levels of Srv. We are currently exploring the relationship between *srv* transcription and the levels of Srv present *in vitro* with plans to expand the work into an *in vivo* model of GAS infection. We are also seeking to determine whether a cofactor exists for Srv.

While the differentially expressed genes were diverse in their putative function, a subset of the genes could be grouped into several general categories:

1. putative or proven virulence factors (Table 3);
2. genes involved in the metabolism, transport, and/or catabolism of nutrients (Table 2);
3. genes involved in translation and posttranslational control (Table 2);
4. genes encoding proven or putative secreted proteins of GAS.

In our previous work, we demonstrated that virulence in MGAS5005 Δ *srv* was significantly reduced in mice in an intraperitoneal model of infection (Reid *et al.*, 2004), a result which agrees with the decrease in virulence factor transcript levels in MGAS5005 Δ *srv*.

A signal P analysis of the genes which were downregulated in the absence of *srv* revealed as many as 51 putative/proven extracellular products may be affected. To test this hypothesis, we conducted a 2D gel analysis of GAS culture supernatant proteins. In each of three replicate experiments, we see a decrease in the production of Sic (Fig. 2), an increase in the production of SpeB (Fig. 2), and an overall increase in the number of extracellular glycolytic enzymes present (data not shown). Despite the absence of an apparent Gram-positive signal secretion sequence, glycolytic enzymes have been previously identified in GAS culture supernatants (Lei *et al.*, 2000; Chaussee *et al.*, 2001), as well as in other organisms (Gozalbo *et al.*, 1998; Modun & Williams, 1999), although an exact mechanism for their release is unknown. In support of this observation, our microarray analysis identified a gene encoding a putative NADP-dependent glyceraldehyde-3-phosphate dehydrogenase as one of the few upregulated genes (SPy1371, 1.79-fold at $A_{600\text{ nm}} = 0.4$).

One observation which is more difficult to reconcile is the early and increased secretion of SpeB. A concomitant increase in caseinolytic activity was also observed, but this is likely due to the increased concentration of SpeB and not to an increase in the proteolytic activity of the SpeB molecule itself. However, several factors known to positively influence SpeB transcription and maturation are down-regulated in the mutant including *mga*, the *dpp* operon, *ropA*, *htrA*, and *luxS* (the degree to which CovRS/CsrRS regulates SpeB is still under debate; Federle *et al.*, 1999; Heath *et al.*, 1999). How do we account for these seemingly disparate results? Evidence indicates that SpeB is produced late in the stationary phase of growth, a time when available nutrients are exhausted (Chaussee *et al.*, 2001). SpeB is responsible for the cleavage of adhesins and/or invasins from the GAS cell surface and the stimulation of inflammatory molecules that may aid in the dissemination of the organism. Our data suggest that allelic replacement of *srv* results in decreased transcription of a large number of genes involved in the transport and metabolism of amino acids and inorganic ions. We hypothesize that GAS interprets this downregulation as entry into a nutrient-poor environment. In fact, sufficient nutrients are available, and growth of the mutant strain is comparable to wild type (Reid *et al.*, 2004), but redundant sensory and metabolic systems are unable to alter the initial

sensory pathway that is directly or indirectly influenced by Srv. Instead, SpeB production is upregulated. Either the activity or the excess presence of SpeB is perceived by the cell, and regulators and accessory molecules related to SpeB production are downregulated in an attempt to reduce SpeB levels. The result *in vivo* is the loss of GAS surface adhesins and/or invasins and limited or unsuccessful colonization. If this hypothesis is correct, and the loss of SpeB temporal control is responsible for the decrease in virulence of the *srv* mutant, the addition of SpeB to log phase cultures should result in decreased GAS virulence in an *in vitro* model of infection. Although an interesting hypothesis, it should be noted that given the number of GAS regulators and virulence factors apparently downregulated in MGAS5005 Δ *srv*, the reduction in virulence is probably attributable to a combination of effects.

In summary, our research suggests that Srv functions as a GAS regulatory unit and is required for production and/or secretion of Sic and SpeB. Our studies were performed using organisms that were cultured in rich medium under optimal growth conditions. Given that we do not yet know the input signals which govern Srv function, analysis of organisms grown under different conditions may yield different results. Furthermore, our studies were limited to the use of a serotype M1 organism. It should not be assumed that the regulatory network controlled by Srv will respond equivalently in other serotypes or strains of GAS. It is reasonable to hypothesize that strain-to-strain variation in gene regulation may account for some of the differences in virulence associated with certain strains. What is clear is that GAS possesses a large number of regulators whose function overlaps or is superimposed in some cases (Kreikemeyer *et al.*, 2003). The systematic study of these systems *in vitro* and *in vivo* will yield new insight into how GAS responds to its environment and how it achieves colonization and transmission.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Correlation of DNA microarray and real-time reverse transcriptase-PCR analysis. The fold changes in transcript of MGAS5005 Δ *srv* relative to MGAS5005 obtained by microarray and real-time reverse transcriptase-PCR were log-transformed and plotted against each other to measure the correlation. The transcription of five genes was compared. Two genes were analyzed from RNA isolated at $A_{600\text{ nm}} = 0.4$, while three separate genes were analyzed from RNA isolated at $A_{600\text{ nm}} = 0.6$.

Fig. 2.

Two-dimensional gel electrophoresis separation of culture supernatant proteins from MGAS5005 (a, c) and the isogenic mutant lacking *srv*, MGAS5005Δ*srv* (b, d). Circled proteins are Sic (a, b) and the zymogen form of SpeB (c, d). There is a marked reduction in the amount of Sic present in the supernatant of MGAS5005Δ*srv* (b) and an increase in the amount of SpeB present (d) compared to the parental strain (c).

Fig. 3.

Casein agar assay for SpeB proteolytic activity. MGAS5005 (a) and MGAS5005Δ*srv* (b) were stab inoculated into agar plates containing skim milk and incubated for 18 h anaerobically. Proteinase 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 MGAS5005Δ*srv* compared to MGAS5005.

Fig. 4. Immunoblot analysis of culture supernatant fluids isolated from MGAS5005 and MGAS5005 Δ *srv* during the exponential phase of growth. Antisera to SpeB was used to analyze 20 μ g of protein isolated from MGAS5005 (lanes 1, 3) and MGAS5005 Δ *srv* (lanes 2, 4) after 2 (lanes 1, 2) and 8 h (lanes 3, 4) of growth. 1.5 μ g of purified SpeB was used as a control (lane 5).

Table 1Genes upregulated ≥ 1.5 -fold in MGAS5005 Δ *srv*

SPy No. and time point when upregulation was observed (M1 or M18* genome)	Gene	Putative/proven function
SPy0067 ($A_{600\text{nm}} = 0.4$)	<i>rplR</i>	50S ribosomal protein L18
SPy0130 ($A_{600\text{nm}} = 0.2$)	NA	Hypothetical
SPy0166 ($A_{600\text{nm}} = 0.4$)	NA	Hypothetical
SPy0724 ($A_{600\text{nm}} = 0.2$)	<i>rplS</i>	50S ribosomal protein L19
SPy1150 ($A_{600\text{nm}} = 0.4$)	<i>nox</i>	NADH oxidase
SPy1371 ($A_{600\text{nm}} = 0.2, 0.4$)	<i>gapN</i>	Glyceraldehyde-3-phosphate dehydrogenase
SPy1531 ($A_{600\text{nm}} = 0.6$)	<i>dpr</i>	Peroxide resistance protein
SPy1687 ($A_{600\text{nm}} = 0.2$)	NA	Hypothetical
SPy1882 ($A_{600\text{nm}} = 0.2$)	<i>lppC</i>	Putative acid phosphatase
SPy2114 ($A_{600\text{nm}} = 0.2$)	NA	Hypothetical
SPyM18-0164	NA	Hypothetical MGAS8232 protein
SPyM18-0261	<i>gapA</i>	MGAS8232 Glyceraldehyde-3-phosphate dehydrogenase

* The array was supplemented with DNA from MGAS8232 (serotype M18) and MGAS315 (serotype M3) representing ORFs believed to be unique in each strain.

Table 2Number of genes downregulated ≥ 1.5 -fold in the *srv* mutant strain compared to wild-type MGAS5005

Code	Description * (no. of genes in each category)	Downregulated at $A_{600\text{nm}} = 0.2$ (% [†])	Downregulated at $A_{600\text{nm}} = 0.4$ (% [†])	Downregulated at $A_{600\text{nm}} = 0.6$ (% [†])
J	Translation (152)		40 (26.3)	34 (22.4)
K	Transcription (133)	1 (0.8)	13 (9.8)	6 (4.5)
L	Replication, recombination and repair (128)		16 (12.5)	13 (10.2)
D	Cell cycle control, mitosis and meiosis (32)	1 (3.1)	2 (6.3)	1 (3.1)
O	Posttranslational modification, protein turnover, chaperones (56)		15 (26.8)	8 (14.3)
M	Cell wall/membrane biogenesis (76)	1 (1.3)	8 (10.5)	9 (11.8)
N	Cell motility (22)		1 (4.5)	3 (13.6)
P	Inorganic ion transport and metabolism (78)		10 (12.8)	8 (10.3)
T	Signal transduction mechanisms (70)		6 (8.6)	3 (4.3)
C	Energy production and conversion (62)		12 (19.4)	7 (11.3)
G	Carbohydrate transport and metabolism (140)		12 (8.6)	14 (10.0)
E	Amino acid transport and metabolism (117)	5 (4.3)	27 (23.1)	22 (18.8)
F	Nucleotide transport and metabolism (65)		9 (13.8)	8 (12.3)
H	Coenzyme transport and metabolism (45)		4 (8.9)	4 (8.9)
I	Lipid transport and metabolism (50)	4 (8.0)	9 (18.0)	4 (8.0)
Q	Secondary metabolites biosynthesis, transport and catabolism (18)	1 (5.6)	7 (38.9)	5 (27.8)
R	General function prediction only (185)		21 (11.4)	16 (8.6)

* Function predicted by the orthologous relationships (COGs) determined for the SF370 serotype M1 GAS genome. Genes that lack a COG assignment or are of unknown function are not included.

[†] Number of genes downregulated ≥ 1.5 -fold in the *srv* mutant strain compared to wild-type MGAS5005. Percent determined as number of downregulated genes vs. total number of genes for each category.

Table 3
Virulence factor genes downregulated ≥ 1.5 -fold in MGAS5005 Δ *srv*

SPy No. (M1 genome)*	Fold change (MGAS5005 Δ <i>srv</i> : MGAS5005)	A_{600nm}	Gene	Putative/proven function
SPy0167	0.50	0.6	<i>slo</i>	Streptolysin O
SPy0212	0.61	0.4	<i>speG</i>	Pyrogenic exotoxin G
SPy0216	0.66	0.4	NA	RofA-related regulatory protein
SPy0334	0.60	0.6	<i>covR/csrR</i>	Control of virulence regulator
SPy0337	0.51	0.4	<i>covS/csrS</i>	Sensory transduction kinase
SPy0416	0.45	0.6	<i>prtS</i>	Putative cell envelope proteinase
SPy0470	0.51/0.61	0.4/0.6	NA	Myosin-cross-reactive antigen
SPy0714	0.63	0.4	<i>adcA</i>	Putative adhesion protein
SPy1054	0.60	0.4	<i>scl2</i>	Collagen-like protein
SPy1159	0.54/0.62	0.4/0.6	<i>hylIII</i>	Putative h aemolysin III
SPy1273	0.60	0.4	<i>cfa</i>	cAMP factor
SPy1357	0.64	0.4	<i>grab</i>	Protein GRAB
SPy1436	0.60	0.6	<i>mf3</i>	Mitogenic factor 3
SPy1896	0.57	0.4	<i>ropA</i>	RopA protein
SPy1979	0.57	0.4	<i>ska</i>	Streptokinase
SPy2007	0.57	0.4	<i>lmb</i>	Laminin adhesion
SPy2010	0.52	0.4	<i>scpA</i>	Streptococcal C5A peptidase
SPy2016	0.49	0.6	<i>sic</i>	Streptococcal inhibitor of complement
SPy2019	0.62/0.53	0.4/0.6	<i>mga</i>	M protein transacting positive regulator
SPy2200	0.66/0.42	0.4/0.6	<i>hasA</i>	Hyaluronate synthetase

* SPy numbers assigned based upon annotation of GAS strain SF370 (Ferretti et al. (2001)).